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The synthesis of deuterated arylamine DNA adducts for use in the development of an isotope dilution LC/MS/MS method

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THE SYNTHESIS OF DEUTERATED ARYLAMINE DNA
ADDUCTS FOR USE IN THE DEVELOPMENT
OF AN ISOTOPE DILUTION LC/MS/MS METHOD

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Chemistry

by
Philip Dmitri Olsen
B.S., University of California Santa Cruz, 1995
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ABSTRACT

Polycyclic aromatic amines (arylamines) are a class of chemical carcinogens that are prevalent in environmental and industrial settings. In order to study the mechanism of their toxicity a quantitative and qualitative detection method was developed to measure the C8-adenine adducts of benzidine and 2-aminofluorene in DNA samples. To do this a novel synthetic method using a palladium catalyst was developed to prepare authentic and deuterated arylamine adducts to serve as standards. These standards were then used to develop a high performance liquid chromatography, electrospray, tandem mass spectrometry, isotope dilution, detection method. To demonstrate the validity of this method two spike and recovery experiments were performed.

CHAPTER 1. INTRODUCTION

1.1 Background and Biological Significance

Nitrogen-containing polycyclic aromatic hydrocarbon (PAHs), including primary aromatic amines, occur intermittently with other PAHs that are ubiquitous in the environment and are prevalent in coal and its refined products.¹ They have been used extensively in the dye, rubber, and plastics industries, and some can be found in tobacco smoke or in cooked foods. Both 2-aminonaphthalene and 4-aminobiphenyl may be found in nanogram quantities in cigarette smoke and additional arylamines may be found in amounts as high as micrograms.² The largest industrial exposure to an arylamine may be to 4,4'-methylenebis(2-chloroaniline) from its widespread use in the polyurethane industry.³ 2-Acetylaminofluorene was initially intended to be used as an insecticide before it was found to be carcinogenic, and now it is the most extensively studied arylamine.⁴ During the cooking process amino acids and other biologically active molecules such as glucose and creatinine are pyrolyzed to form potent heterocyclic arylamine mutagens. Some nitroaromatic hydrocarbons, found in diesel emissions and other combustion products, such as wood smoke, are metabolized differently but damage DNA by an almost identical mechanism as arylamines. The structures of some of these compounds are presented in Figure 1. These discoveries have led to extensive research on the mutagenicity and carcinogenicity of arylamines, including their metabolic activation and the mechanisms of their toxicity.

Chemical carcinogenesis was first recognized over two hundred years ago in 1761, when an increase in nasal polyps was associated with an increased prolonged use of tobacco snuff.⁵ Fifteen years later Percival Pott noted an increase in scrotal skin cancer

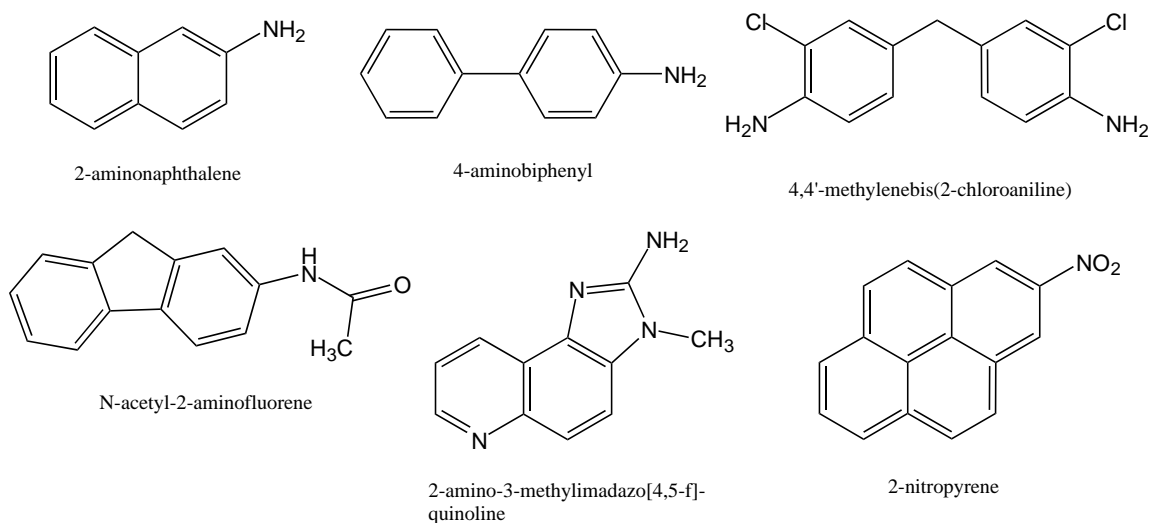


Figure 1. Structures of representative carcinogenic aromatic nitrogen compounds

among chimney sweeps, and determined that it was due to years of heavy exposure to coal soot and tar.⁶ Polycyclic aromatic amines, or arylamines have been suspected carcinogens since 1895, when Rehn documented several cases of bladder cancer among dye manufacturers, which he attributed to aniline.⁷ This was later determined to be from exposure to the dye precursors of aminonaphthylenes and benzidine instead.⁸ The rubber antioxidant, 4-aminobiphenyl has also been found to increase the formation of bladder tumors in workers who have been exposed to it.⁹

Arylamines have indeed been found to be carcinogenic in a variety of mammals, primarily at sites associated with their metabolism. Some of this data is summarized in Table 1. From this table it can be seen that most of these amines form tumors in the bladders of humans and dogs but in the livers of rodents. This is due to metabolic activation differences that vary from species to species. It is not the actual arylamines that causes genetic damage but activated metabolites that form covalent bonds to DNA bases. Ironically some of the same pathways that lead to the detoxification and excretion of some toxic substances may also lead to the formation or activation of other

compounds. Arylamines are activated *in vivo* by a variety of enzymes that oxidize and/or acetylate the amine functional group,

Table 1. Site and Species of Some Carcinogenic Arylamines

Compound	Target of Tumorigenesis	Species
Benzidine	Bladder	Humans, Dogs
	Liver	Rats, Mice, Hamsters
4-Aminobiphenyl	Bladder	Humans, Dogs, Mice
	Liver	Mice
	Mammary gland	Rats
2-Aminonaphthalene	Bladder	Humans, Monkeys, Dogs, Rabbits, Guinea pigs, Rats, Hamsters
	Liver	Mice
2-Acetylaminofluorene	Bladder	Dogs, Rabbits, Mice
	Liver	Dogs, Rats

that goes on to form an electrophile that can covalently bond with certain bases present in DNA.⁴ These bound adducts may then be responsible for mutations in the form of base substitutions or deletions in the DNA sequence that could lead to cancer. The primary intermediate of the activation process is an N-hydroxyaryamine that is then activated enzymatically, or may simply be activated by protonation under acidic conditions.

Although each arylamine is metabolized slightly differently, the generalized process is outlined in Figure 2.

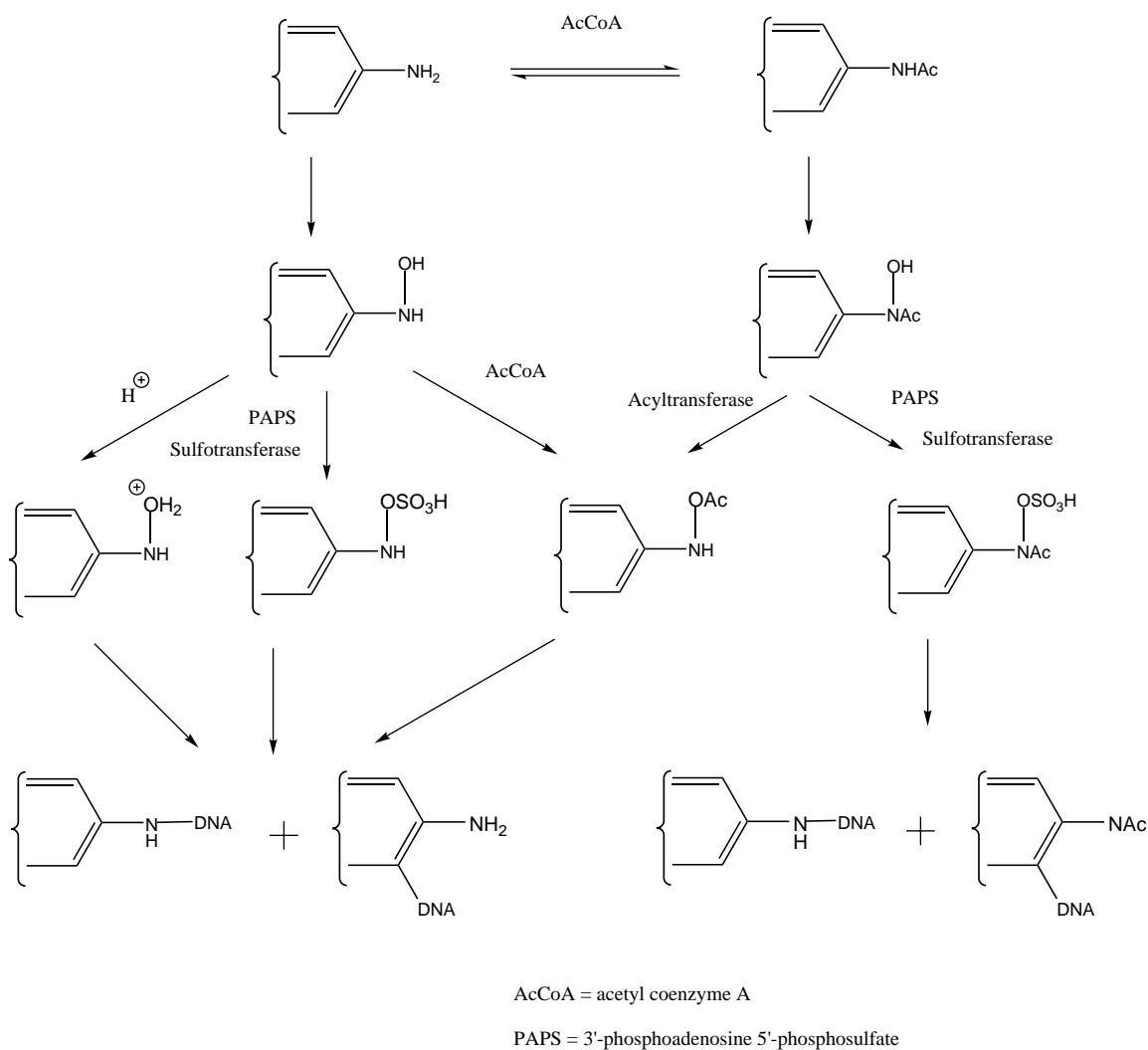


Figure 2. The metabolic activation of arylamines

Because many of the oxidative enzymes are found in the liver, but tumors form in the bladder, the arylamines may be oxidized in the liver and then transported to the bladder via glucuronidation.² It is also possible that they could actually be oxidized in the bladder by peroxidases found there such as prostaglandin *H* synthase.¹⁰ Unlike 2-aminonaphthalene, 1-aminonaphthalene is not carcinogenic, but its oxidized form N-

hydroxy-1-aminonaphthalene is strongly carcinogenic.² This presents evidence that unlike its isomer, 1-aminonaphthalene is not a suitable substrate for oxidation. In the case of arylamides, oxidation alone is not sufficient for activation of this class of chemicals, and they require either acyltransferase or sulfotransferase to form a reactive electrophile.¹¹ Often when N-acetylarylamines are administered to animals most of the adducts detected are not acetylated. This is the case with 4-acetylamino-biphenyl and 2-acetylamino-fluorene.² N-acetylbenzidine is not deacetylated, but the primary adduct that forms is linked through the non-acetylated amine.⁴ While the role of acetylation in metabolic activation is not entirely understood, a statistical correlation was found between human bladder cancer from industrial exposure to arylamines, and the slow acetylator phenotype of the polymorphic NAT2 N-acetyltransferase gene.¹² Apparently certain sub-populations may be at a greater risk from arylamine induced cancer than others due to differences in their metabolic profiles. This observation was not substantiated, however, in a later study that monitored only benzidine exposure and not a mixture of arylamines.¹³ After activation to form a reactive electrophile, the arylamine bonds to the nucleophilic sites of DNA.

The purpose of our research was to develop a detection method that is suitable to study arylamine adduct formation in both *in vitro* and *in vivo* systems. In order to do this it was required that we synthesize authentic and deuterated arylamine DNA adducts to be used as standards. Once fully characterized we desired to use these standards in a high performance liquid chromatography (HPLC), tandem mass spectrometry (MS/MS) detection method. It was also our objective to demonstrate the validity and efficiency of this method.

CHAPTER 2. LITERATURE REVIEW

2.1 Arylamine Reaction Mechanisms with DNA

The most nucleophilic site among bases found in DNA is the N7 position of guanine, which is also the site where most alkylating carcinogens bond as well.^(K94) It was originally thought that the activated electrophile, most likely a nitrenium ion, forms an N7-arylamine adducts that is unstable and deprotonates. This creates an ylide intermediate that undergoes a Stevens' rearrangement to form the most prevalent C8-deoxyguanosine adducts.¹⁴ This process is outlined in Figure 3.

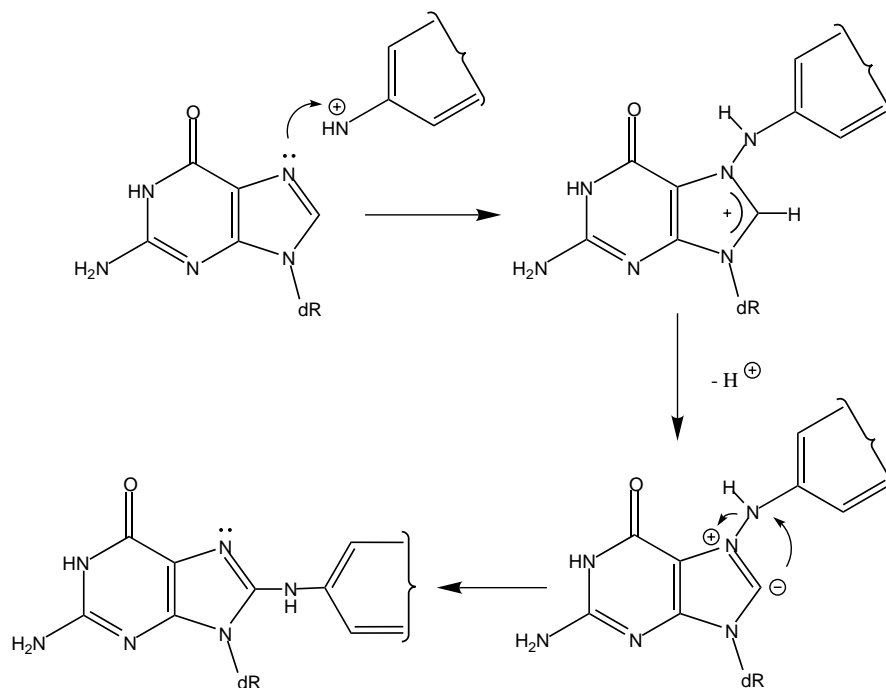


Figure 3. Stevens' type N7 rearrangement

Evidence for N7 attack by a nitrenium ion is provided by a study in which C8,N9-dimethylguanine was reacted with N-hydroxylaminofluorene to produce a stable N7-guanyl adduct.¹⁵ NMR data from this report indicates coupling through the aminofluorene nitrogen and the presence of a split C8 methyl group and split C8 proton

excluding the possibility for C8 coupling. Conversely, in a different study where 8-methylguanosine was reacted with N-acetyl,N-acetoxyaminobiphenyl, the researchers reported characterizing a diastereomeric C8-guanyl adduct.¹⁶ They retain the idea of an initial reaction at N7 but propose a different rearrangement reaction to form the C8 adduct by means of an alternate resonant structure. This mechanism can be seen in Figure 4.

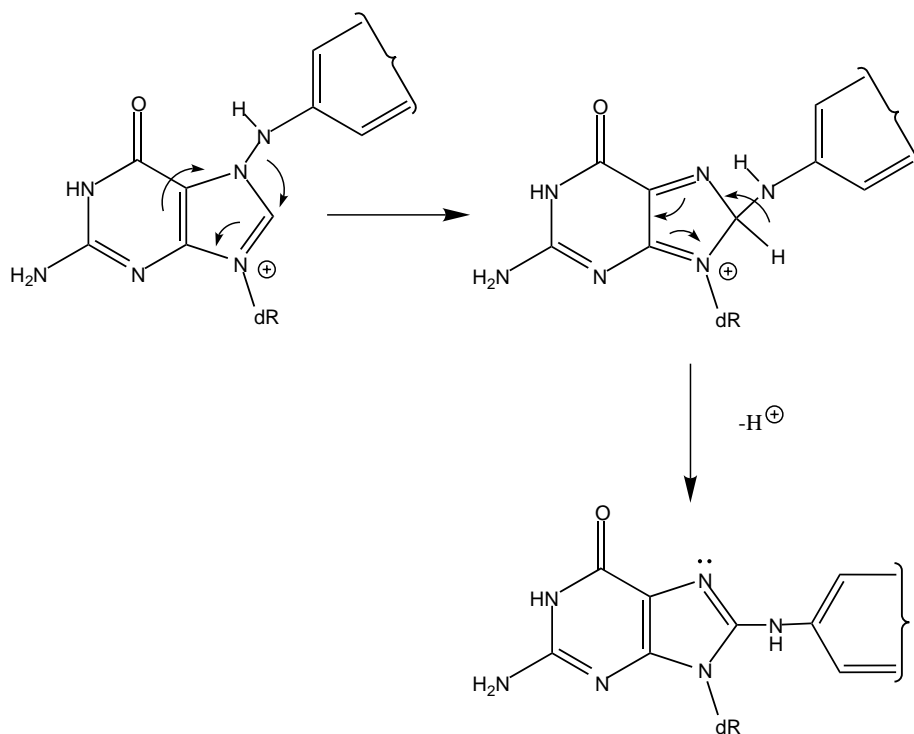


Figure 4. An alternate N7 to C8 rearrangement

It is conceivable in the first study that it was possible to isolate the N7 adduct because a less electron withdrawing methyl group replaced the usual deoxyribose. The fact that different arylamines were used in each study may have also contributed to the different results. Recently, it has been reported that nitrenium ions bond directly to the C8 position of guanine, utilizing laser flash photolysis and kinetic data.¹⁷ These authors reacted the nitrenium ions of 2-aminofluorene and various 4-aminobiphenyls with

solutions of deoxyguanosine. They report that the kinetics for the formation of an ylide does not match their data for the rate of C8 deprotonation, and that an equilibrium constant for a migration would be unreasonable, while the kinetics of the 4-amino,4'-methoxybiphenyl nitrenium ion show an inverse secondary isotope effect, indicative of a carbon changing from sp^2 to sp^3 hybridization. Their only comment on the low nucleophilicity of C8 is that the intermediate ion is very stable, analogous to the intermediates for the electrophilic substitution of benzene derivatives. Regardless of the mechanism, the most prevalent type of adducts that form *in vivo* are C8 guanine adducts, but other types of adducts form as well.

Other nucleophilic sites on bases in DNA that are capable of forming adducts are the C8, and N6 positions of adenine, and the N2, and O6 of positions guanine. While the C8 adenine adducts are presumably formed by a similar mechanism to the C8 guanine adducts, the other adducts most often formed by nucleophilic addition of the base to a resonance form of the nitrenium ion, where the charge has moved to a carbon, usually alpha, to the nitrogen substituent. These types of adducts are formed by linkage of the purine nitrogen to an arylamine carbon, with the exception of 1-N-hydroxy-aminonaphthalene. This compound forms both the arylamine N-O linked adduct, N-(deoxyguanosin-O6-yl)-1-aminonaphthylene and the arylamine C-O linked adduct, 2-(deoxyguanosin-O6-yl)-1-aminonaphthylene.² The formation reactions of these types of adducts is seen in Figure 5. This is partially explained by Huckel molecular orbital-based perturbation theory that predicts that the 1-aminonaphthalene nitrenium ion should have more S_N1 character than any other arylamine ion.¹⁸ Semiempirical AM1 molecular orbital calculations have been used to predict which arylamine carbon is likely to be

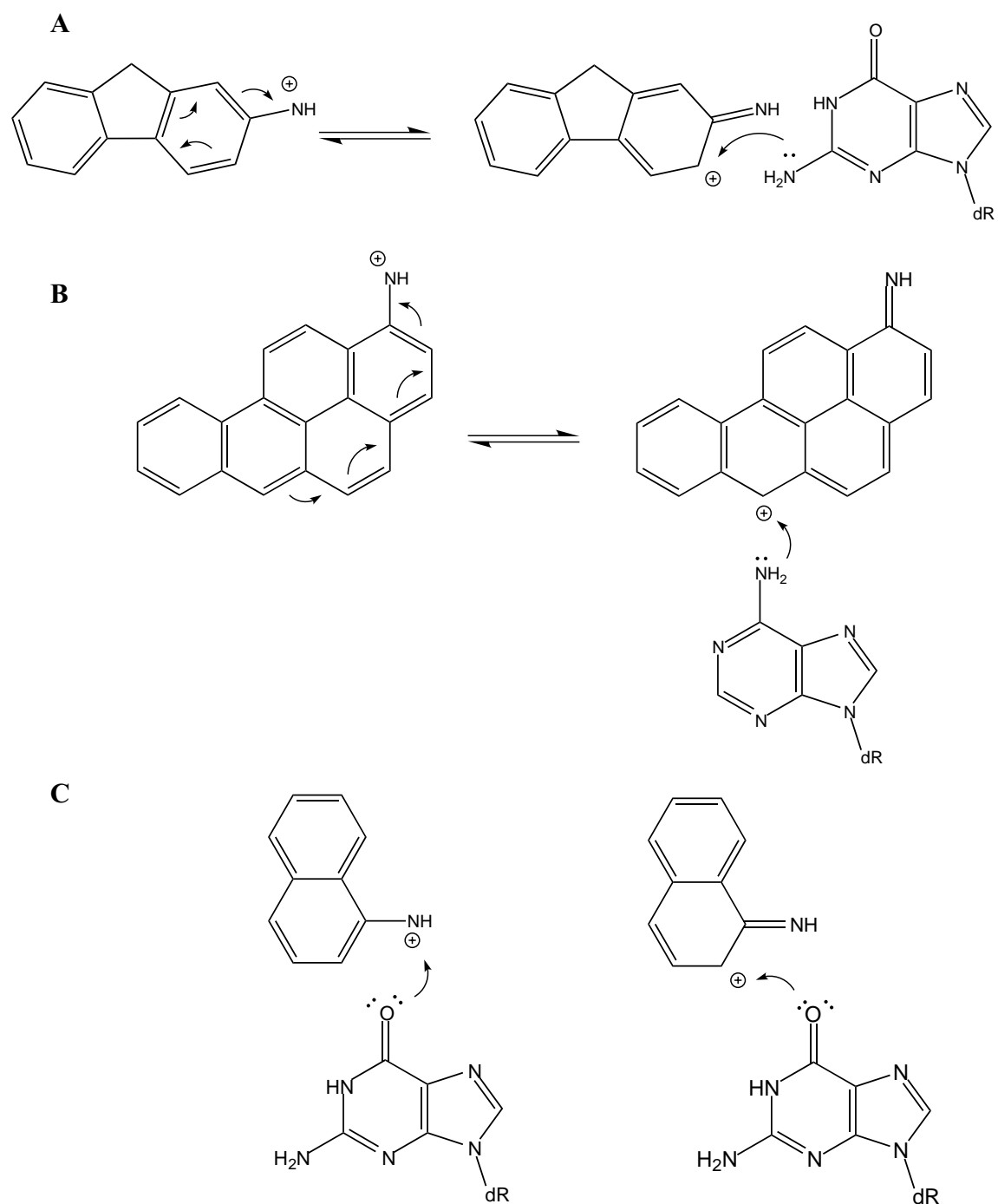


Figure 5. N2 dG, N6 dA, and O6 dG adduct formation with (A) 2-aminofluorene, (B) 1-aminobenzo(a)pyrene, and (C) 1-aminonaphthalene

attacked, and while in some cases it is the carbon next to the substitution, for some larger more conjugated amines such as 1-aminobenzo(a)pyrene, the most likely spot for reaction may be as far as five carbons away.¹⁹

It has also been proposed that some type of stacking orientation between the nucleoside and the arylamine may contribute to the position of adduct formation.²⁰ The different types of adducts formed *in vivo* for several arylamines are presented in Table 2.

Table 2. Structural Characterization of Arylamine Adducts.

Arylamine	C8-dG	C8-dA	N2-dG	N6-dA	O6-dG
Benzidine	Major				
4-Amino-biphenyl	Major	Minor	Minor C3 linked	Minor C3 linked	
1-Amino-naphthalene	Minor				Major N linked Minor C2 linked
2-Amino-naphthalene	Major		Minor C1 linked	Minor C1 linked	
2-Amino-fluorene	Major		Minor C3 linked		
4,4'-Methylene-bis(2-chloro-aniline)		Major			

After the adduct has formed, its structure has a profound effect on its biological fate. Both the structure of the arylamine and the location of its binding can determine its persistence and consequences in an organism. Acetylated 2-aminofluorene adducts are not found in dogs and are much less persistent in rats than the non-acetylated form because the acetylated form causes the rotation of guanine around its glycosal bond

which adopts a *syn* conformation that significantly distorts DNA and may signal preferential repair.¹¹ This is confirmed by NMR studies of acetylated and non-acetylated aminofluorene-dG adducts, which led to the conclusion that the non-acetylated adduct was less distorting, and would reside in the major groove based on space filling models.²¹ Conversely a recent solution conformation study of an 11-mer oligonucleotide duplex containing a non-acetylated 2-aminofluorene C8-dG adduct opposite a deletion site, found that the arylamine intercalates into the deletion site and that the guanine is modified to the *syn* alignment.²² The presence of the deletion site and the brevity of the 11-mer oligonucleotide may explain why these results differ from earlier data.

Seven days after a single dosing of 2-aminonaphthalene, C8-dG adducts can still be found in the bladders of dogs, where tumors occur, but the adducts can no longer be found in the liver where tumors are rare.²³ The prevalence of this adduct suggests that it is the 2-aminonaphthalene C8-dG adduct that is responsible for mutations. This same study also found that the most potent bladder carcinogen in dog, 4-aminobiphenyl, was also formed the most persistent adducts with almost no loss of C8-dG, N2-dG, or C8-dA adducts occurring over one week. However, these adducts also persisted in the liver of dogs where 4-aminobiphenyl does not cause tumors. From this it seems apparent that overall binding and persistence of DNA adducts is important to carcinogenicity, but are not the only factors that matter. It could be possible that it is actually the rarely formed C8-dA adduct that accounts for 4-aminobiphenyl's potency as a bladder carcinogen. It has been proposed that it is the increase in dA binding that accounts for the increased carcinogenicity of dimethyl-benzo(a)pyrene over its unsubstituted version.²⁴ These authors further theorize that dA adducts may be potent because they could cause

mutations in the TATA box involved in initiating transcription, or the AT rich sequence that regulates the *c-mos* oncogene. Irregardless of adenine adduct significance, the more abundant C8-dG adducts have been found to cause base pair substitutions and deletions in both bacteria and mammalian systems.¹⁴

The most studied C8-dG arylamine adducts are those of 2-aminofluorene. 2-aminofluorene and N-acetyl-2-aminofluorene modified *NarI* restriction enzyme recognition sequences were found to cause mutations in bacteriophage M13mp9.²⁵ In this system the non-acetylated adduct caused G to T transversions and G to A transitions, while the acetylated adduct caused CG deletions. N-hydroxy-N-acetyl-2-aminofluorene has been found to cause CG to AT transversions in the first base of codon 61 of *Ha-ras*, GC to TA transversions in the first base of codon 13 of *N-ras*, and CG to GC transversions in the first base of codon 13 of *Ki-ras* in CD-1 mice.²⁶ Several oligonucleotides were modified with 2-aminofluorene to test its effect on the enzymes involved in DNA repair. Synthetic primer templates with acetylated and non-acetylated adducts caused an increase chance of incorrect dATP incorporation by T7 DNA polymerase opposite and immediately after the adduct.²⁷ A 2-aminofluorene adduct on a transcribed strand caused a weak pause by RNA polymerase II, and an acetylated adduct caused an absolute block.²⁸ Either type of adduct located on the nontranscribed strand enhanced polymerase arrest at a nearby sequence specific pause. The heterodimer hMutS α , which participates in strand-specific mismatch repair, specifically binds to both types of 2-aminofluorene adducts as well.²⁹ Differences in the types of mutation brought about by the acetylated or nonacetylated adducts are attributed to their conformational

structures. It is commonly thought that sequence around the adduct and the repair system of the organism effect the type of mutations that occur.

2.2 Synthesis of Arylamine Adducts

In order to study the mechanism of arylamine carcinogenicity, their DNA adducts have been prepared synthetically so that they could be characterized structurally, and used as standard references in qualitative and quantitative detection methods. As mentioned previously, they have also been prepared in order to measure their effect on different enzymes. This has usually been done by reacting an activated form of the arylamine, with either whole DNA, an oligonucleotide, or single nucleosides. These methods are limited however, because the activated forms of the arylamines are often unstable, they react in low yield, and extensive purification is often required. Consequently, alternate synthetic methods have been developed. One of these methods creatively employs a base analog and multiple steps to synthesize C8-G adducts, while other approaches rely on nucleophilic substitution of protected 8-bromoguanosine by the arylamine. Recently these types of substitution reactions have been greatly improved by the use of palladium catalysts.

2.2.1 Adduct Synthesis by Activated Arylamine Pathways

Traditionally DNA adducts have been prepared by methods that mimic their biological formation. Here, the activated arylamine, usually a hydroxy or acetoxy arylamine, is incubated with some source of DNA bases in an acidic buffer solution around pH 5, that produces the same type of adducts *in vitro* that are produced by *in vivo* exposure. Hydroxyl amines will not usually react with single nucleosides and nucleotides, but they will react *in vitro* with whole DNA.¹⁴ Acetoxy amines will react

with nucleosides and nucleotides but react faster with DNA. This is attributed to electrostatic attraction between the activated nitrenium ion and the anionic phosphate backbone of DNA.³⁰ Also, acetoxy amines will usually form C8G adducts exclusively with single bases and a mixture of adducts with DNA.¹⁶ This further supports the idea that the structure of DNA itself is important for adduct formation *in vivo*. The hydroxy and acetoxy compounds are most often made by partial reduction and acetylation of the corresponding nitro compound. The three most common routes for nitro reduction and acetylation are outlined in Figure 6.

The first hydroxylamines of this type were synthesized by the general method developed by Willstatter and Kubli in 1908.³¹ This method uses a saturated solution of NH_3 and H_2S to reduce the corresponding nitro compound to a hydroxylamine. This procedure was adapted and used to synthesize N-hydroxy-2-aminofluorene in 1963,³² and then slightly improved two years later by replacing a difficult anaerobic extraction with precipitation and centrifugation.³³ Average yields of hydroxyl amine were 65%. Several studies have used this method^{21,34,35,36} to prepare N-hydroxy-2-aminofluorene that was then reacted with deoxyguanosine, deoxyguanosine 5'-phosphate, a G containing decamer, or whole DNA. Yields of adducts for the nucleoside and nucleotide were 15 and 5% respectively, after recrystallization with 2:1 isopropyl alcohol/water. Yields of the decamer were similar after purification by reverse-phase C_{18} HPLC. The reaction performed with whole DNA yielded roughly one adduct per 15 bases after enzymatic hydrolysis, Sephadex LH20 chromatography, and reverse-phase C_{18} HPLC.

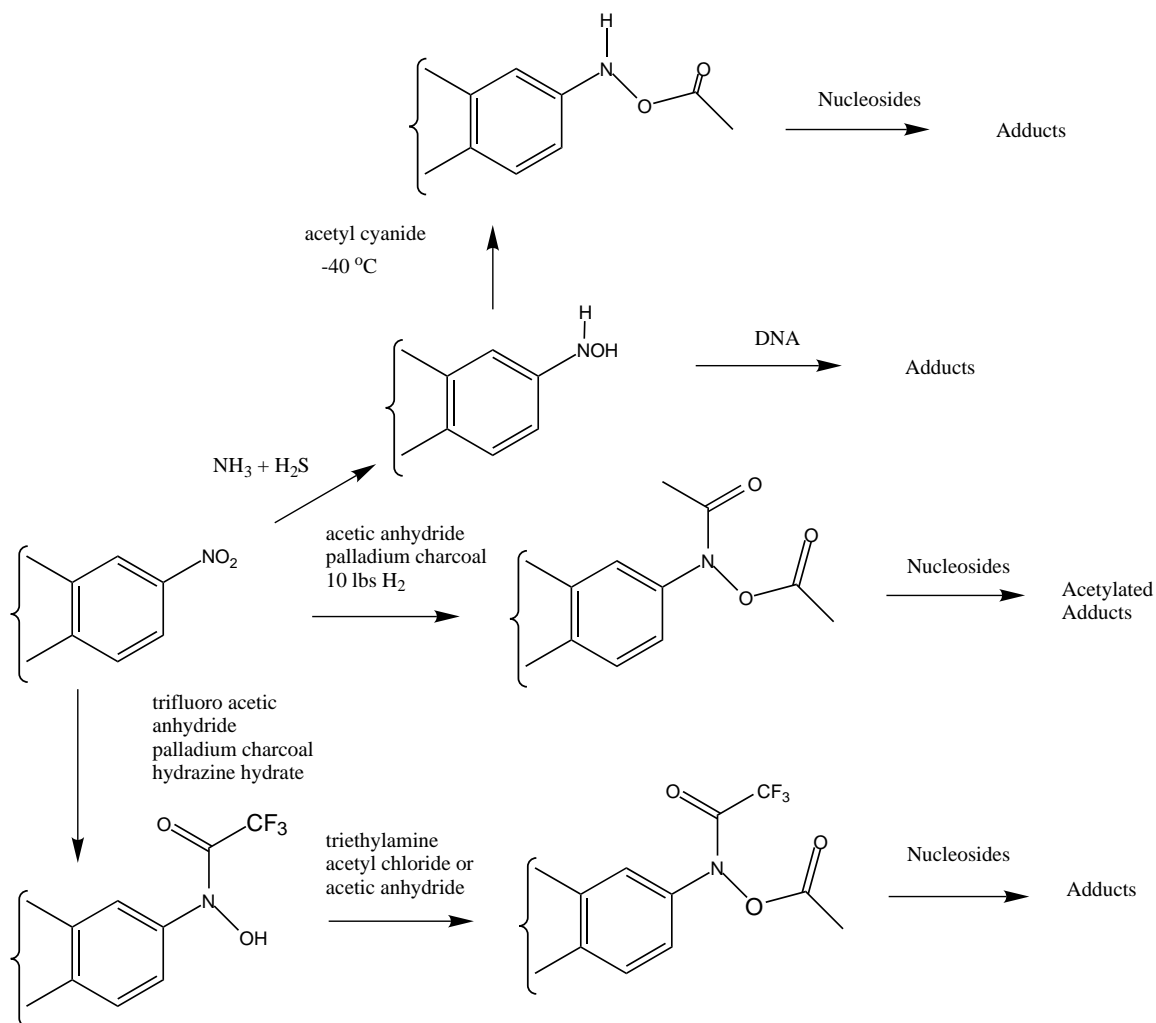


Figure 6. The reduction and acetylation of nitro compounds

An alternate method of reduction uses hydrogenation with palladium charcoal and acetic anhydride in ethyl acetate.³⁷ This method will produce either an acetoxy or hydroxy-amide. If the hydroxy-amide is desired rather than the acetoxy-amide, 1N NaOH in 25% ethanol can be used to perform the hydrolysis. The reported yield of hydroxy-amide by this method is only 20-30%. This method has been used to prepare N-hydroxy-N-acetyl-2-aminofluorene that was reacted with deoxyguanosine to produce adducts that were then used to synthesize site modified oligonucleotides.^{29,38} Yields of

adducts in these studies were not mentioned, but the oligonucleotides were prepared on the pmol. scale.

A similar reduction also uses palladium charcoal, but with hydrazine hydrate as opposed to hydrogenation.³⁹ Here the nitro compound is dissolved in tetrahydrofuran with palladium charcoal at 0 °C and hydrazine hydrate is slowly added. Hydroxyl amines may be isolated by filtering to remove the catalyst, washing with sodium acetate buffer, and then drying with MgSO₄ followed by evaporation of the organic solvent. Many hydroxyl amines are extremely sensitive to aerobic oxidation and must be handled in an inert atmosphere. Hydroxy-amides are prepared by adding triethylamine, then acetyl chloride in tetrahydrofuran prior to work up by neutralization with NaHCO₃, extraction with 0.1 M NaOH, washing with ether, and then precipitating with concentrated HCl. Yields for this method are reported as 65-88% depending on nitro compound used. This method was used to prepare hydroxyl amines and amides of 4-aminobiphenyl, 4,4'-methylenedianiline, and 2-aminofluorene.^{25,40,41,42} These hydroxyl amines and amides were then reacted with different types of bases to produce adducts in less than 5% yield after extensive HPLC purification. One paper in particular used hydroxyl amines prepared in this way to synthesize adducts in a relatively high yield.⁴³ A solution of N-hydroxy-aminofluorene (0.1mM) was incubated with dG (5mM) in a 10 mM potassium citrate, 0.1 mM EDTA buffer in the presence of acetylsalicylic acid (10m M). The acetylsalicylic acid reacts with the hydroxylamine at neutral pH to form the non-acetylated adduct in 84% yield measured by HPLC. This yield is equal to that of the reaction done without acetylsalicylic acid at pH 5. As the reaction was done on a 1.5 ml scale, roughly 60 µg of adduct was produced.

Depending on the type of adducts desired, N- and O- acetylation can be incorporated into the nitro reduction, or O- acetylation can be performed on an isolated hydroxyl amine or amide. O- acetylation can be directly performed on a hydroxyl amine with acetyl cyanide at -40°C in tetrahydrofuran.⁴⁴ The reaction of hydroxyl amines with acetic anhydrides at 0°C produces the corresponding N-acetyl, O-acetoxy amine. It is also possible to do the first N-acetylation with trifluoroacetic anhydride and then the O-acetylation with acetic anhydride and the resulting adducts produced from this material and nucleosides will be non-acetylated.^{25,42,45} Theoretically, any gentle reductive method that will not reduce an aromatic nitro compound completely to an amine could be used. One reference formed N-hydroxy-1-aminopyrene *in situ* from its nitroso form during the DNA incubation by including ascorbic acid. Numerous references have produced DNA adducts by these methods using hydroxy and acetoxy compounds obtained as gifts or from commercial sources.^{22,27,46,47,48}

These reductive methods activate the nitro compounds by converting them to a reactive species that goes on to form the DNA adducts. It is generally agreed that the reactive species is a nitrenium ion.³⁰ The hydroxy and acetoxy amines generate this nitrenium ion in acidic solutions by a dissociative process called solvolysis but they can also be made photochemically from the corresponding azide.^{17,40} All synthetic methods that go through this route are plagued by difficulties in generating an unstable oxidation state and then further difficulties by competitive side reactions of the nitrenium ion. Consequently, overall yields are low, less than 5% on average, and extensive purification, usually by HPLC, is required.

2.2.2 Alternate DNA Adduct Synthetic Pathways

Because of the low yield of adducts prepared directly from nitrenium ions, other types of syntheses have been explored. The most involved of these is a multistep method that couples modified arylamines with base analogs and then converts them to N-(purin-8-yl)arylamine adducts. The other synthetic method used to prepare C8-G arylamine adducts does so by the nucleophilic substitution of 8-bromoguanosine 2', 3', 5'-triacetate. While each method has been used with limited success, each has its own limitations.

The multistep synthetic method has been used to prepare C8-G and C8-A adducts of the ortho, meta, and para isomers of toluidine also known as methylaniline.⁴⁹ Guanine adducts are made by reacting 6-hydroxy-2,4,5-triaminopyrimidine sulfate with either the dithiocarbamate or isothiocyanate of toluidine to form a thiourea. This compound is then either oxidized with HgO to create a carbodiimide, or methylated with CH₃I to create a methylisothiuronium ion. The carbodiimide and methyl-isothiuronium ion can then both be cyclized thermally to create the final adduct. This scheme is outlined in Figure 7.

Adenine adducts have been prepared by using 4,5,6-triaminopyrimidine sulfate instead of 6-hydroxy-2,4,5-triaminopyrimidine sulfate. Overall yields were reported of approximately 12% using HgO, and 38% using CH₃I. The authors did not address the selectivity of the amine in the 5 position of the pyrimidine compounds to form the thiourea but they did characterize their compounds by NMR and did not report any side reactions. Although these authors prepared roughly 25 mg of each adduct and suggested that their method would be applicable to higher arylamine homologs, there is little if any evidence in the literature for other successful uses of this method.

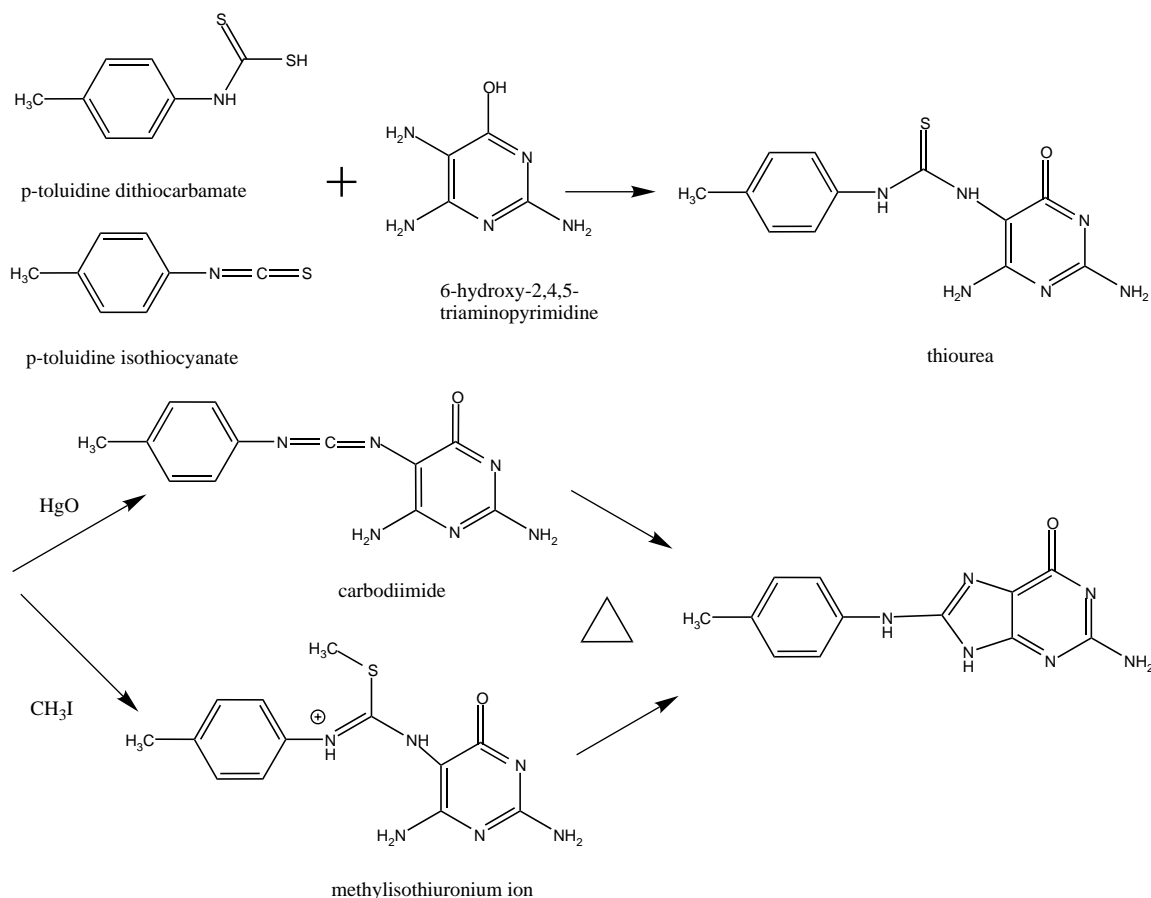


Figure 7. Multistep synthesis of C8-G adduct

Nucleophilic substitution reactions have been used to prepare C8-G adducts of both aniline and 2-aminofluorene on a relatively large scale. This method was first used in 1967 to prepare C8-G 2-aminofluorene adducts.³⁴ In this approach, 8-bromoguanosine 2',3',5'-triacetate and 2-aminofluorene in isopropyl alcohol, were sealed in a thick walled glass tube and heated to 150 °C for 20 hours. The tube was opened and the precipitate that formed was hydrolyzed to the base adduct with 1:1 ethanol/1N HCL. The adduct was then precipitated by adding aqueous 1N HCl and recrystallized with 9:1 95% ethanol/concentrated HCl for a yield of 23%. This method was then employed to make C8-G adducts of aniline because they could not be made from any form of hydroxy or

acetoxo aniline.⁵⁰ These authors discovered that depurination would occur above 100 °C and that it was unavoidable with the less stable acetylated 8-bromodeoxyguanosine. They further found that 8-bromoguanine was unreactive alone, but that the addition of HBr or the HBr salt of the arylamine catalyzed the triacetyl-8-bromoguanosine reaction. This was presumably due to protonation of the N7 of guanosine activating it to both substitution and glycosyl cleavage. Rather than hydrolyze to the guanine adduct with acid these authors deacetylated it in 1:1 methanol/0.2 M NaOH. This improved HBr salt method was then used to synthesize the C8-G adduct of 2-aminofluorene in roughly 60% yield.⁵¹ These authors also deacetylated under basic conditions but then utilizing a variety of protective chemistry converted the guanosine adduct to the deoxyguanosine adduct greatly reducing the overall yield to only 5-8%. Nonetheless, they prepared gram quantities of adduct. Their reaction scheme is presented in Figure 8. These same authors later demonstrated that all forms of C8-G arylamine adducts will decompose in aerobic alkali conditions therefore, alkali hydrolysis is inappropriate for isolation of arylamine DNA adducts *in vivo*.(J94,Shinyap3)

In order to prepare large amounts of C8 adducts both the multistep and substitution methods have been used. The multistep method has been used for both C8-G and C8-A adducts of toluidine, with overall yield less than 40%, but only produces free base adducts without a ribose or deoxyribose present. This method does not appear often in the literature as well, possibly indicating that it is difficult to repeat. The substitution method has been used to produce C8 triacetylated guanosine adducts of aniline and 2-aminofluorene in good yield. These adducts could then be deacetylated, also in high yield, but conversion to deoxyguanosine adducts greatly reduced the yield. Compared to

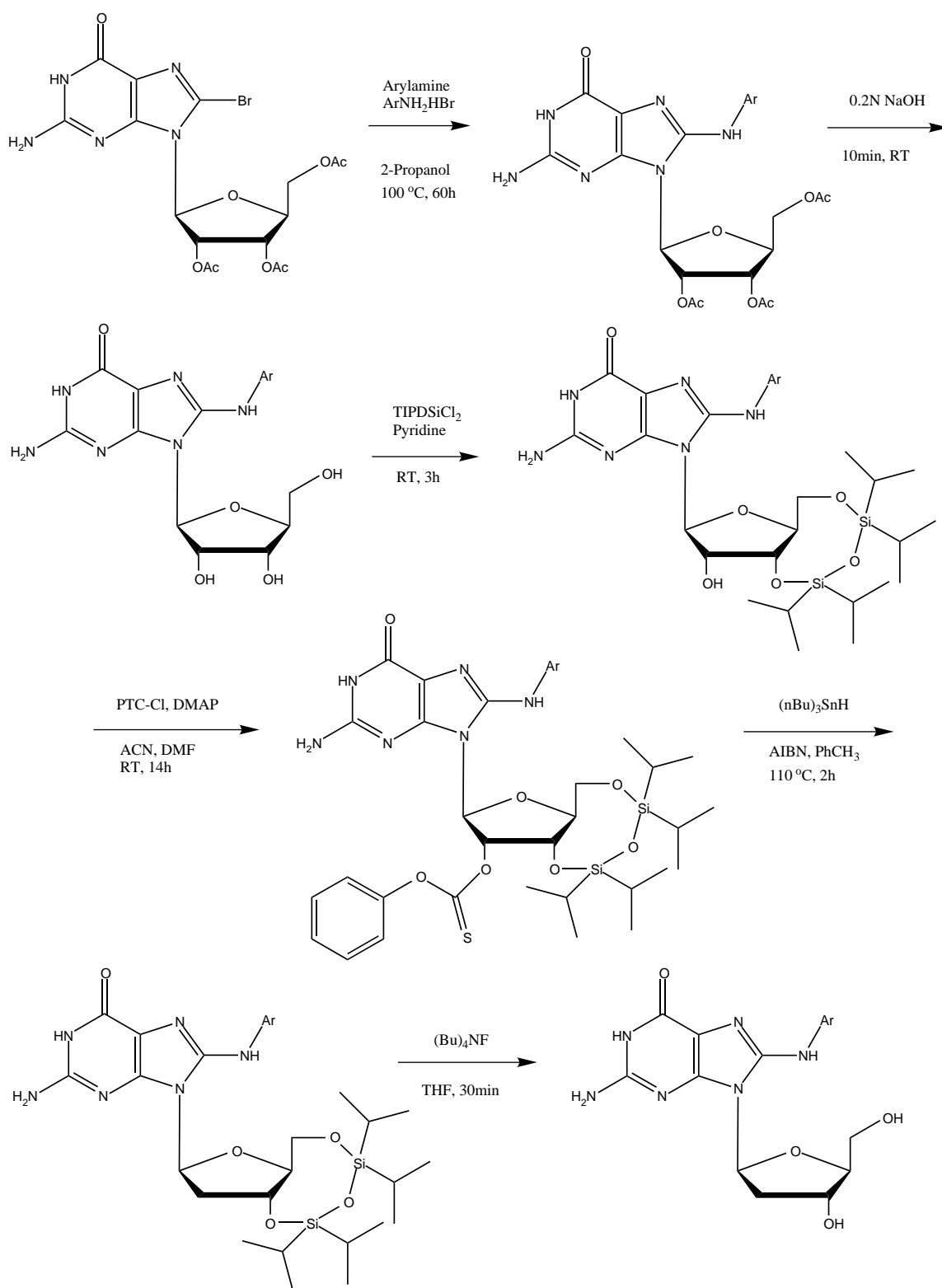


Figure 8. Adduct synthesis with HBr arylamine salt

activated intermediate syntheses these methods offer advantages, but they themselves are still plagued with difficulties.

2.2.3 Adduct Synthesis by Palladium Catalysis

In the last two years several attempts have been made to improve on the substitution method by using palladium catalysts. N2-G, N6-A, and more recently C8-G adducts have been prepared by carbon-nitrogen bond formation using the Buchwald/Hartwig catalytic system. When factoring in the protective chemistry overall yields are around 40% but the methods are reliable and work for a variety of arylamines including some prone to difficult adduct synthesis.

The Buchwald/Hartwig catalysis has numerous applications for the formation of carbon-nitrogen bonds.^{52,53} In general, a palladium complex is used to catalyze the substitution of an aryl halide or triflate (trifluoromethanesulfonyl) with a primary or secondary amine. The source of palladium is usually Pd(OAc)₂ (palladium acetate) or Pd₂dba₃ (tris(dibenzylideneacetone)dipalladium), and while numerous ligands have been used to create the catalysts, the most common used have been DPPF (1,1'-bis-(diphenylphosphanyl)ferrocene) and BINAP (2,2'-bis(diphenylphosphino)-1,1'-binaphthyl). The catalytic cycle is presented in Figure 9.

Once the chelated palladium catalyst has been formed oxidative addition of the aryl halide occurs. The amine then joins the complex, followed by removal of the halide anion with base, and reductive elimination of the product to regenerate the catalytic species. It has been found that if Pd(OAc)₂ is used as a source of palladium it must be premixed with BINAP before addition of the base or formation of the catalyst will be low

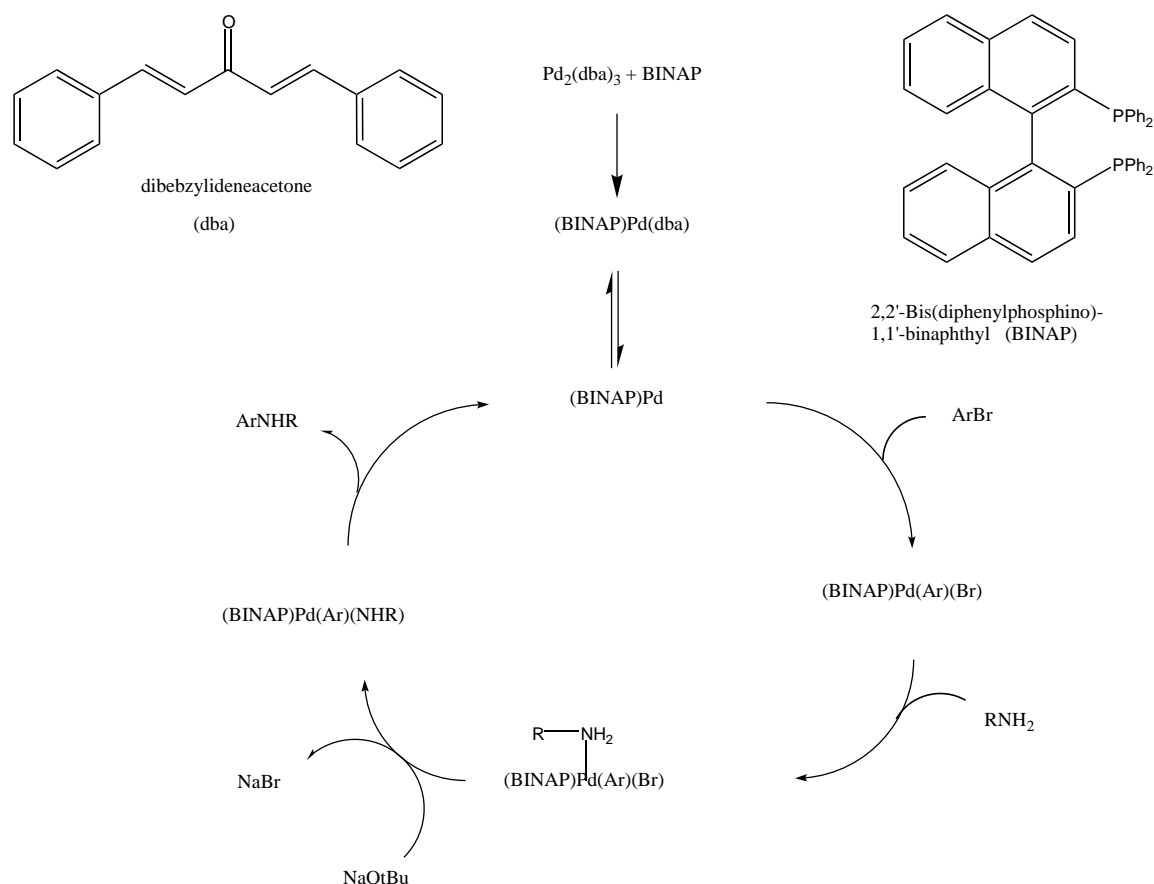


Figure 9. The Buchwald/Hartwig catalytic cycle

leading to slow poor yield reactions.⁵⁴ Specific application of this catalytic method for the synthesis of arylamine adducts did not occur until 1999.

The first adducts prepared using a palladium catalyst were a series of N6 deoxyadenosine analogues made from reacting protected 6-bromodeoxyadenosine with several arylamines including 2-aminofluorene and 4-aminobiphenyl.⁵⁵ The resulting adducts that formed were not really arylamine adducts but N6 aryl adducts that are not found *in vivo*. From examining the literature, they found a new electron rich ligand reported to accelerate oxidative addition of aryl heteroatom halides.⁵⁶ This ligand, 2-dicyclohexylphosphino-2'-(N,N-dimethylamino)biphenyl gave improved yields over BINAP. These authors also found that K_3PO_4 was an ideal base to use as NaOtBu caused

decomposition and Cs_2CO_3 resulted in decreased reaction rates. This method is presented in Figure 10. Yields for the different adducts ranged from 52-72%.

N2 Deoxyguanosine and N6 deoxyadenosine adducts of 2-aminonaphthalene, 2-aminofluorene, 4-aminobiphenyl, and o-toluidine were prepared by reacting the exocyclic amine of a purine with a bromine or triflate strategically placed on an aromatic nitro compound and then reducing.⁵⁷ These authors found no problems with using Cs_2CO_3 as the base or BINAP as the ligand, but noted that O6 benzylation of guanosine was required. They reported overall yields for coupling through deprotection of 70-88% for the most part. Their reaction scheme is presented in Figure 11. In some cases the coupling was so strong that two halides or triflates would join to the purine amine.

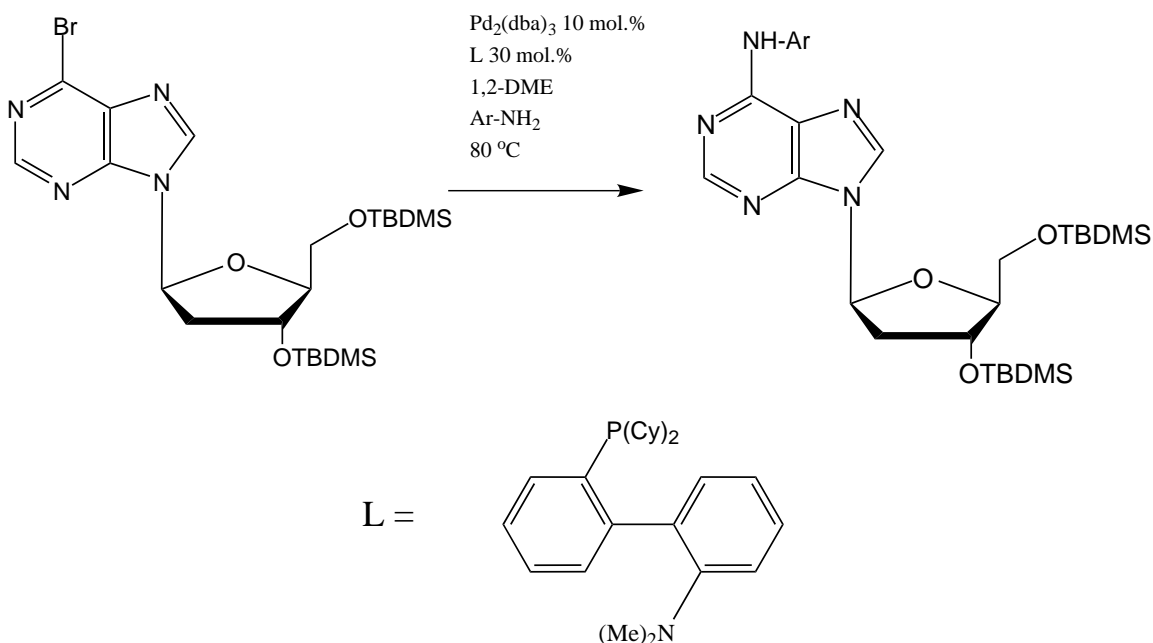


Figure 10. N6 Aryl adduct synthesis with palladium catalyst

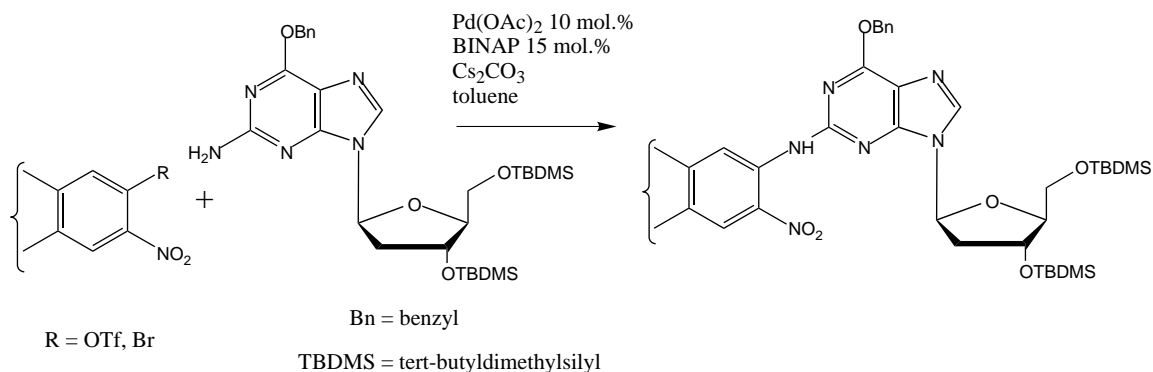


Figure 11. N2 Arylamine adduct synthesis with palladium catalyst

Personal communication with Dr. Johnson confirmed that they had no success coupling either protected 8-amino-deoxyguanosine with 2-bromofluorene, or protected 8-bromo-deoxyguanosine with 2-aminofluorene.

More recently C8-deoxyguanosine adducts for several arylamines have been prepared.⁵⁸ These authors were able to synthesize the C8-deoxyguanosine adduct of the food mutagen IQ (2-amino-3-methylimidazo[4,5-f]-quinoline), a compound not amenable to other types of syntheses, in 32% overall yield from 8-bromo deoxyguanosine. In the case of IQ, the reaction was optimized by protecting both the carbonyl and amine of 8-bromodeoxyguanosine and using the strong base LiHMDS (lithium hexamethyldisilazide). They also prepared protected C8-deoxyguanosine adducts of 4-aminobiphenyl, 2-aminofluorene, and 2-aminonaphthalene in 50-60% with slightly different conditions. In this case they found amine protection as a bis-BOC better than the use of STABASE (tetramethyldisilylazacyclopentane) and that K_3PO_4 was a better base than LiHMDS. They found no difference in yield whether using BINAP or the ligand 2-dicyclohexylphosphino-2'-(N,N-dimethylamino)biphenyl for the arylamines. Their reaction scheme is outlined in Figure 12.

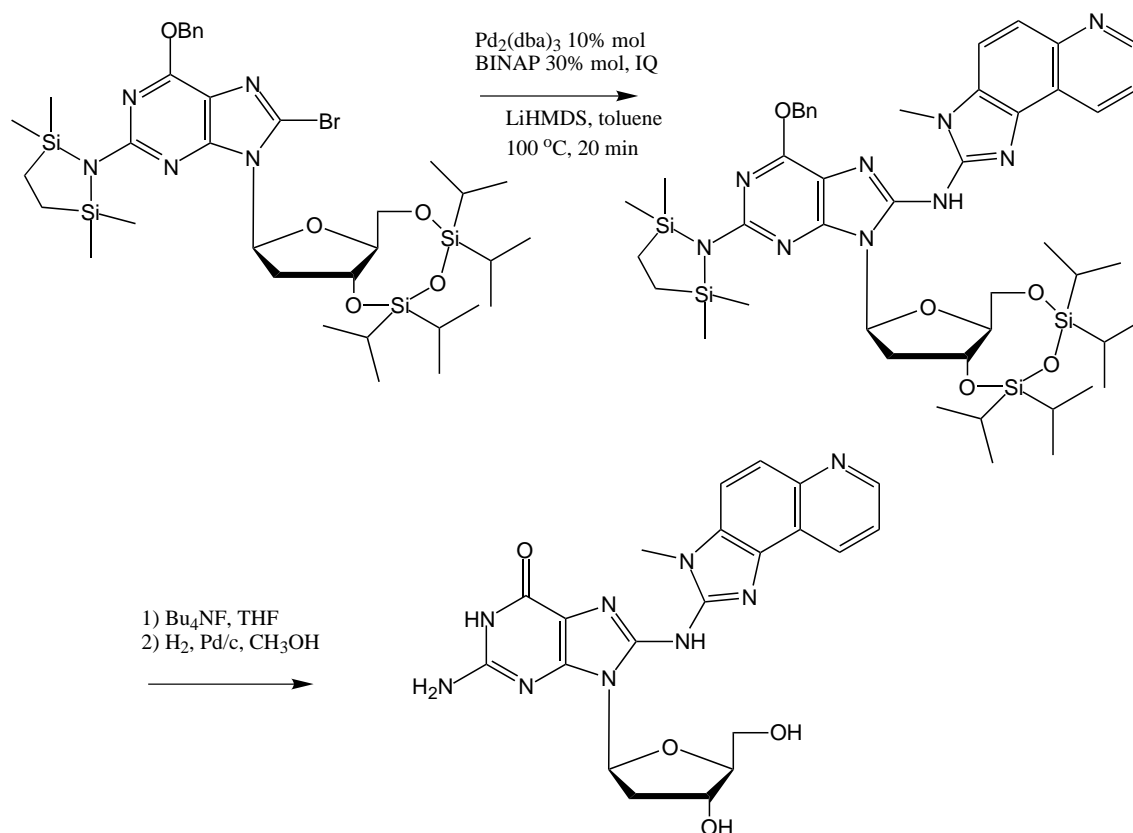


Figure 12. Synthesis of IQ adduct with palladium catalyst

These recent papers utilizing palladium catalysts offer improved yields and ease over previous methods for the synthesis of arylamine adducts. They are also capable of making both C8 and N2 deoxyguanosine adducts as well as N6 deoxyadenosine adducts.

2.3 Detection and Quantitation of DNA Adducts

Because adduct formation occurs at extremely low levels *in vivo*, 1 adduct per 10^6 - 10^8 bases, extremely sensitive detection methods are necessary.²³ Consequently detection has mostly been achieved by radiolabeled methods, immunoassays, or with growing popularity mass spectrometry. All methods require some form of DNA isolation, hydrolysis, and separation to resolve the different adducts from relatively small tissue samples. Thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), capillary electrophoreses (CE), and even gas chromatography

(GC) have all been used. Each method has its own strengths and weaknesses and some references have even compared different methods to judge how well they perform on the same samples.

Radiolabeled methods involve measuring the adducts formed from ^{14}C or ^3H radiolabeled adduct forming compounds or by using the ^{32}P postlabeling method. When ^{14}C or ^3H adducts are formed they are usually analyzed by HPLC with non-labeled synthetic, co-eluting standards added in large amounts detectable by UV. All fractions are collected and measured for radioactivity by scintillation, and the different adducts are identified by their retention time compared to that of the characterized standards. This is sometimes referred to as co-chromatography. This method is limited by the high cost of labeled compounds, the low specific activities of ^{14}C and ^3H , and the necessity of large amounts of characterized standards for co-chromatography. The use of radio labeled compounds may lead to confusing results due to lack of structural information. In one case, C8-G adducts of 4,4'-methylene-dianiline were prepared and the ^{14}C labeled compound was administered to rats at three different dosages.⁴¹ Radioactivity was associated with the hydrolyzed DNA but it did not elute in the fraction that eluted with the guanosine standard. It is likely that only C8-adenosine adducts form *in vivo* as they do for 4,4'-methylenebis(2-chloroaniline).⁵⁹ In another study, [2,2'- ^3H]-N-hydroxy-4-aminobiphenyl was reacted with calf thymus DNA *in vitro* at various concentrations.⁶⁰ Radioactivity of the hydrolyzed DNA was then used as a reference to evaluate a ^{32}P method, an immunoassay, and an MS method. They found that at lower concentrations adduct formation was disproportionately less than at higher concentrations but excess radioactivity was still associated with the DNA as a non-covalently bound degradation

product. This impurity was not found in the high concentration reactions or *in vivo* and is not believed to be an adduct, but this demonstrates how because this method is not qualitative false results can be obtained by measuring total radioactivity.

The other commonly used radiolabeled method for measuring DNA adducts is the ^{32}P postlabeling method. Here the adducted DNA is hydrolyzed to 3'-monophosphate nucleotides, usually with micrococcal nuclease and spleen phosphodiesterase, and then T4-polynucleotide kinase and $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ is used to 5'-phosphorylate the nucleotides with radiolabeled phosphate. The next step is to separate the few labeled adducts from the vast excess abundance of labeled non adducts. Sometimes this is eased by the fact that T4-polynucleotide kinase will preferentially labels some adducts over regular nucleotides. If the adduct is significantly hydrophobic, preliminary purification can be done by C18 filtration, where normal nucleotides are removed first with water and then the adducts are eluted with methanol. Alternately, the adducts can be enriched by extraction into *n*-butanol. Final purification is then done by reversed-phase HPLC, or more commonly by two-dimensional TLC on polyethylene-imine-cellulose plates. Radioactivity is measured by scintillation counting of fractions for HPLC, or by autoradiography for TLC. Sometimes Nuclease P1 is used to remove the 3'-phosphate from the nucleotides, heightening the difference between the adducts and other nucleotides, but this reportedly undesirably dephosphorylates C8 purine adducts.^{61,62} Although this method can be extremely sensitive due to the high specific activity of ^{32}P it is completely dependent on the efficiency of the hydrolysis, butanol extraction, and labeling phosphorylation. Often additional experiments are required to optimize these steps.^{41,63} In the previously mentioned 4-aminobiphenyl study the ^{32}P postlabeling

method significantly underestimated the amount of adducts, even compared to the high concentration standards that did not contain extra radioactivity.⁶⁰ HPLC has been used in a ³²P postlabeling method for the adduct of several arylamines.⁶⁴ These authors report fast analysis times at the cost of resolution compared to TLC. Overall postlabeling is very sensitive and does not require large amounts of sample. Then again it does require radioactive material, it is only partially qualitative by chromatography, and it is susceptible to low recoveries.

A few types of immunoassays have been used to detect arylamine adducts. All require that antibodies be prepared that recognize the desired DNA adducts. An antiserum was developed that detects deoxyguanosin-8-yl-acetylaminofluorene and deoxyguanosin-8-yl-aminofluorene but does not recognize N-acetyl-2-aminofluorene, or any minor adducts. This serum has been used in a radioimmunoassay (RIA)⁶⁵ and an enzyme-linked immunosorbent assay (ELISA).⁶⁶ The RIA works by mixing the sample with the antibody and a radioactive standard. When the antibody antigen complex is isolated, the sample concentration is measured by the loss of radioactivity due to the sample displacing the labeled standard. For the ELISA test, the antigen is linked to a polymeric support and the sample and antibody are added and then rinsed. In the absence of sample, all antibody will bind to the standard, when it is present a proportional amount will be removed in the wash. The amount of bound antibody is determined by a second antibody that binds to the first, but is also attached to an enzyme that produces colored or fluorescent products. The previously mentioned 4-aminobiphenyl study used a dissociation-enhanced lanthanide fluoroimmunoassay (DELFI) with rabbit anti-N-(guanosin-8-yl)-2-acetylaminofluorene. This antibody successfully recognized the 4-

aminobiphenyl adduct. This type of assay is like an ELISA but has biotin bound to the second antibody in place of an enzyme. It then uses the strongly fluorescent europium-labeled streptavidin that binds to biotin to measure the amount of original antibody that bonded to the fixed antigen. These authors found that the assay overestimated the amount of adducts formed. They attributed this to the antiserum reacting more strongly with the *in vivo* deoxyguanosine adducts than it did with their synthetic guanosine standards. Immunoassays have excellent sensitivity but require antibodies that are difficult to prepare. They also are so specific that they are not able to detect multiple adducts, or so non-specific that they are not able to distinguish between adducts, and possibly interferences.

Mass spectral methods have been performed with GC, HPLC, and CE used for separation. In the specific case of arylamine adducts, GC requires hydrolysis of the adducts with base to release the arylamines, that are then extracted, derivitized, and introduced into the GC. The isolated DNA can be extracted before hydrolysis to insure that no unbound arylamines are present. This method has been used to detect 4-aminobiphenyl adducts from the bladder and lung mucosa of human smokers,⁴⁶ and in the 4,4'-methylenedianiline study mentioned previously. Recoveries from the hydrolysis were 55 to 85%, and no structural forms of adducts could be identified because only the arylamine is detected. This was fortunate in the case of the 4,4'-methylenedianiline study because they expected deoxyguanosine adducts and did not find any, but they were able to determine that some type of adduct had formed. Both methods used deuterated arylamines as internal standards and confirmed their results by the ³²P postlabeling method. Drawbacks of this method include the fact that it is not qualitative, there is

relatively low recovery from the hydrolysis, and it is dependent on the efficiency of the derivitization. Hydrolysis to the arylamine is required for GC because the whole adducts cannot be volatilized into the gas phase. HPLC and CE on the other hand may be used to analyze much larger nonvolatile compounds.

The 4-aminobiphenyl study used a complicated HPLC system with two pumps, a trap column and an analytical column, and switches that directed the flow to either a waste reservoir or a single quadrupole mass spectrometer. Two columns were used in order to concentrate the sample on the trap column at a high flow rate, before loading it on to the analytical column, which operated at a low flow rate so as to not overwhelm the mass spectrometer. The sampling skimmer-cone was switch between low and high voltage to produce in-source collision-induced dissociation and the selected ions of the 4-aminobiphenyl adduct, its deuterated standard counterpart, and their largest fragments were monitored. Detection of a molecular ion and a characteristic fragment ions offer good confirmation of the identity of the analyte. Different isomers of adducts may also be identified by their different fragmentation patterns, such as in the case of the C8-G and N2-G adduct of 2-aminofluorene that were measured by capillary chromatography coupled with continuous-flow fast atom bombardment tandem mass spectrometry.⁶⁷ The major fragment of the C8 adduct has a m/z of 165 and a unique secondary fragment with m/z of 140 is also produced, while for the N2 adduct a major fragment with a m/z of 180 and a unique secondary fragment with a m/z of 222 is produced. In tandem mass spectrometry, two mass spectrometers are used in series. The first mass filter passes only the desired parent ions and the second selects only the desired fragments or daughter ions. With triple quadrupole mass spectrometers the middle set of quadrupoles does not

scan but is used as a collision cell to produce the fragments detected by the last set of quadrupoles. Selected ion monitoring (SIM) or multiple reaction monitoring (MRM) that only detect desired ions, offer increased sensitivity by removing background ions, thus increasing the signal to noise ratio, and by increasing scanning time on only one or few specific masses. This type of scanning typically makes possible adduct detection at levels as low as 30-50 fmol.⁶⁸ Because deoxynucleoside adducts usually lose their deoxyribose when they fragment, constant neutral loss (CNL) experiments can also be performed. In CNL scanning, all ions are detected that come from parent ions that have lost a specific fragment, in this case a 116 dalton deoxyribose group. This method does not offer increased sensitivity over full scan mode but it does simplify the resulting mass chromatogram, although non-adducted nucleosides are also detected. This type of scanning has been used to characterize an *in vitro* reaction of calf thymus DNA and N-acetoxy-N-acetyl-2-aminofluorene.⁶⁷

This method also used CE for separation with sample stacking to improve sensitivity. CE methods offer better separation resolution than HPLC by having less peak broadening and more theoretical plates for analyte interaction. Sample stacking involves concentrating dilute solutions on the front of the capillary before separation. In this case it was discovered that a 350nl injection of a 5×10^{-6} M solution of adducts was equal to a 35nl injection of a 5×10^{-5} M solution, or a 3.5nl injection of a 5×10^{-4} M solution. Capillary electrochromatography (CEC) has been coupled with electrospray ionization to detect the DNA adducts of benzo(g)chrysene and 2-aminofluorene as well.⁶⁹ These authors demonstrated how stopping the electroosmotic flow mid run did not cause peak broadening from diffusion, and mentioned that this would be ideal for ion traps where

long data acquisition times are required to detect fragments of fragments of fragments.

Of all the methods mentioned, only mass spectrometry provides direct qualitative information, and with sample concentrating and MRM comparable detection limits to the other types of methods can be achieved.

CHAPTER 3. THE SYNTHESIS OF ARYLAMINE DNA ADDUCTS

3.1 Introduction and Objective

The overall goal of this project was to develop an HPLC, electrospray, tandem mass spectrometry, quantitative detection method. In order to achieve this it was necessary to synthesize deuterated and non-deuterated DNA adducts of selected arylamines to be used as internal standards and for the construction of a quantitative calibration curve. The synthesis of arylamine DNA adducts was attempted by numerous routes, before a new method was developed based on work that was done with Buchwald/Hartwig palladium catalyst. This method was successful for preparing the C8-adenine adducts of several arylamines, in a few steps, and on a large enough scale for their easy characterization by NMR. In order to lessen the cost of using deuterated arylamines various labeling and synthetic routes from inexpensive starting materials were also explored.

3.2 Preparation of Deuterated Arylamines

The synthesis of deuterated arylamine adducts required that large amounts of deuterated arylamines be available for coupling. As even stable isotopically labeled compounds are expensive, an inexpensive and efficient route for their preparation was sought. 2-aminoanthracene (2AA) was the arylamine we were primarily interested in, as it is similar enough to known carcinogenic arylamines, to suggest a similar toxicity, but it is much less well studied. It is also slightly more bulky than 2-aminonaphthalene, indicating that its DNA adducts might be more interfering and thus more mutagenic.

The first method attempted was an adaptation of the tritiation procedure developed by Yavorsky and Gorin⁷⁰ which had been used successfully on aromatic

insecticides,⁷¹ coal by-products,⁷² and even arylamines.⁷³ This procedure involves reacting tritiated water with phosphorus pentoxide (P_2O_5) to make tritiated phosphoric acid, and then saturating the acid with BF_3 gas to make a more reactive reagent. The compound to be labeled is then introduced in a small amount of solvent that has no exchangeable protons. Aside from acidic protons, only the aromatic protons on carbon are exchanged by this method. For our purposes, deuterium oxide (D_2O) was used instead of tritiated water, as a compound with an altered mass was desired rather than a radioactively labeled one.

Attempts to deuterate 2AA by this method were unsuccessful. After work up, analysis by HPLC and APCI MS indicated only a 10% increase in the M+1 ion intensity and a 3% increase in the M+2 ion intensity. There was also evidence of extensive decomposition to heavier products, especially after prolonged exposure to light and oxygen. One of these impurities, isolated by HPLC/MS had an uncommon isotopic pattern ranging from 209 to 215 m/z compared to that of 194 for the $[M+H]^+$ ion of 2AA. This could conceivably be a partially deuterated, partially oxidized form of 2AA such as 2-nitrosoanthracene or 2-amino-9-anthracenone. The other impurity had a m/z of 481 and is likely to be some type of oxidized dimer. Performing the reaction without a cosolvent or at higher temperature only increased the amounts of impurities formed. While a roughly 10% labeling efficiency may be suitable for tritiation, successful deuteration requires a complete exchange of at least three protons to increase the mass above naturally occurring isotopes.

The next method attempted for the deuteration of 2AA was the high temperature dilute acid (HTDA) method developed by N.H. Werstiuk.^{74,75} This involves labeling

aromatic hydrocarbons with dilute DCl /D₂O (2-4%) at temperatures between 250-280 °C in evacuated and sealed thick walled glass tubes. Werstiuk deuterated 97% of the aromatic protons on the hydrochloride salt of aniline by repeating this method several times on the same material. He also found that at temperatures above 250 °C the aniline would decompose to phenol. In our hands with 2AA this procedure caused almost complete formation of the 481 m/z impurity found earlier with small amounts of unlabeled 2AA remaining. 2-nitroanthracene was also tried and also decomposed for the most part. GC/MS of the nitro reaction revealed that one of the impurities that formed had a broad isotopic pattern centered at 293 m/z. This is 70 m/z greater than the mass of 2-nitroanthracene (223 m/z), indicating that in addition to side reactions some deuteration was occurring.

It is believed that the mechanism of deuterium exchange for these methods is similar to electrophilic aromatic substitution, where cationic intermediates are formed. While amines are considered activating for aromatic substitution, in the presence of acid they are protonated themselves, forming a cation that would discourage the addition of any more deuterium atoms essential for exchange. This is portrayed in Figure 13. This problem was eventually overcome by the use of a platinum catalyst.

A method developed for the deuterium labeling of aromatic amino acids⁷⁶ was modified for use with arylamines. Here Pt⁰ or Adams catalyst is mixed with D₂O and the amino acid to be labeled is added in a solution of 40% deuterated sodium hydroxide (NaOD). The mixture is then refluxed under argon for 48 hours. Because 2AA is not

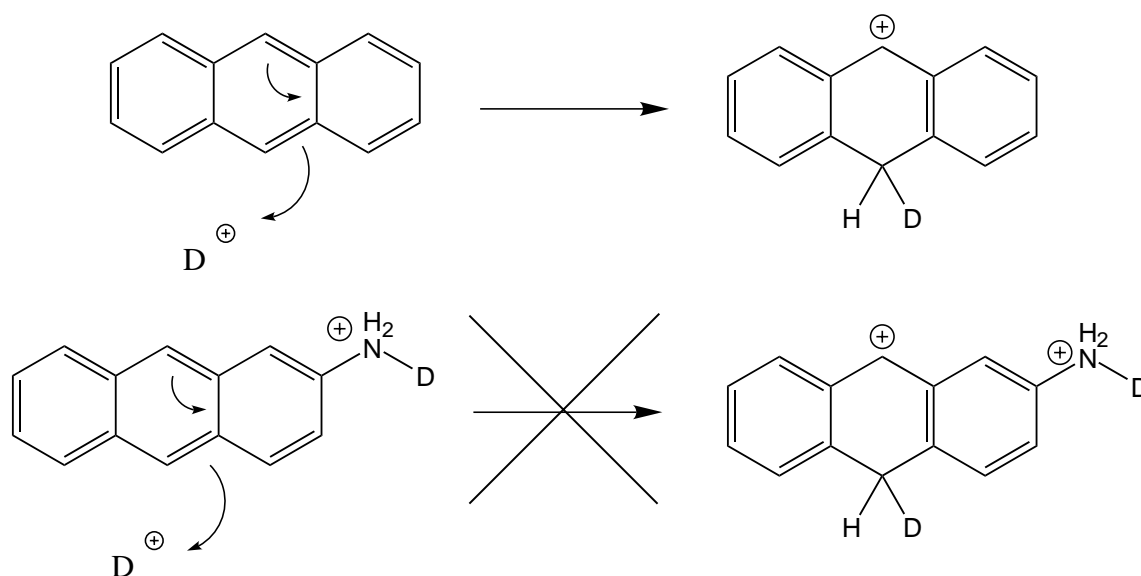
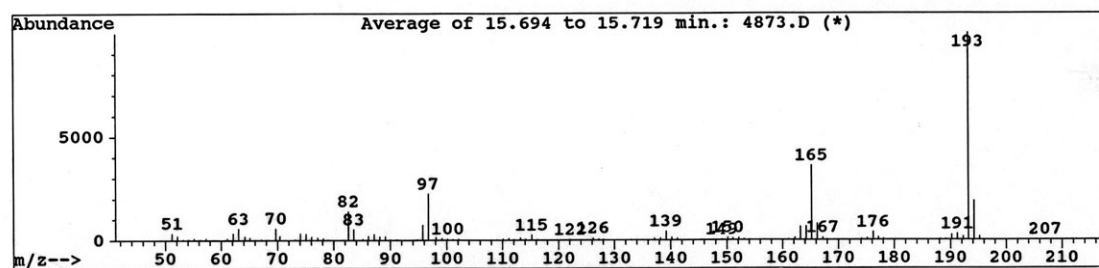


Figure 13. Deuterium exchange of 2-aminoanthracene

soluble in NaOD deuterated ethanol (EtOD) was used instead. After five sequential exchanges, 2AA was obtained with an isotopic pattern corresponding to a 52% exchange of seven protons with the most intense isotope being $M+4$, determined by GC/MS. A GC/MS mass spectrum of deuterated 2AA and an unlabeled 2AA reference standard are presented in Figure 14.

Compared with substituted compounds, deuterated polycyclic aromatic hydrocarbons are inexpensive, and it was thought that they could be used as precursors to labeled arylamines. Fluorene is preferential nitrated in the two position and then easily reduced to its corresponding arylamine with zinc.⁷⁷ This procedure was tried first with nonlabeled fluorene and then repeated with a different reduction,⁷⁸ using per-deuterated fluorene to synthesize d_9 2-aminofluorene in 46% overall yield from d_{10} fluorene. Ironically the new reduction used hydrazine hydrate and palladium carbon in refluxing ethanol. These conditions are very similar to those used to reduce nitro compounds to hydroxy amines, except that the temperature is kept at 0 °C rather than at a 79 °C reflux.

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 Quality : 94
 ID : 2-Anthracenamine



File : C:\HPCHEM1\1\DATA\6112.D
 Operator : Olsen
 Acquired : 13 Nov 98 2:46 pm using AcqMethod DB5
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 Vial Number: 1

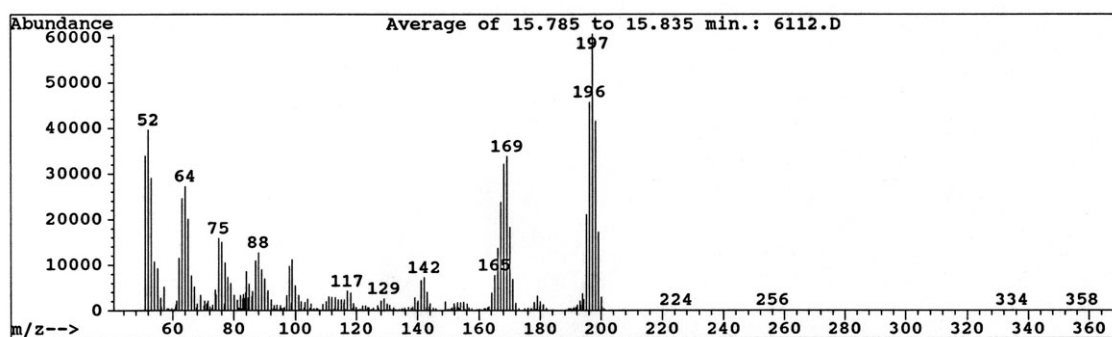


Figure 14. Mass spectrum of labeled 2-aminoanthracene and standard

This same procedure has been used at least twice before in the literature to prepare d_9 2-aminofluorene.^{79,80}

Unfortunately in the case of anthracene the two positions on its central ring are highly activated towards substitution by resonance. This is because if substitution occurs at one of these positions the two exterior rings still maintain their aromaticity during formation of the intermediate. This is illustrated in Figure 15. Consequently 9-nitroanthracene is the only product formed upon nitration. To prevent nitration at this position an attempt was made to nitrate 9,10-dihydroanthracene by the same method used to nitrate fluorene. Because the 9,10 positions would no longer be aromatic they would not be available for nitration. The product could then be easily rearomatized by

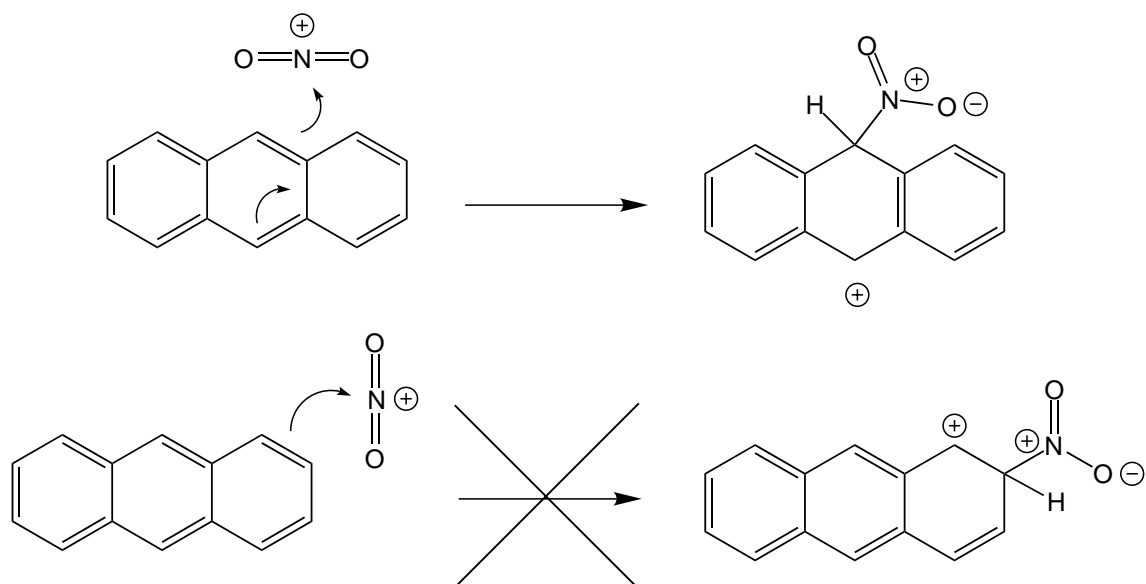


Figure 15. The nitration of anthracene

dehydrogenation. GC/MS of the reaction revealed a mixture of three components. The primary component produced was 9,10-anthracenedione with lesser amounts of 9-anthracenone, and 9-nitroanthracene also present. Apparently the nitric acid was oxidizing and rearomatizing the 9,10-dihydroanthracene prior to nitration.

3.3 Adduct Synthesis by Activated Arylamine

Although it was initially avoided due to the risks involved with handling an activated carcinogen, it was eventually decided to attempt this method, as no others seemed to work. This method requires that nitro compounds be reduced and or acetylated and then incubated with a source of nucleosides. As 2-nitroanthracene is not commercially available it was prepared by the oxidation of 2-aminoanthracene, which is. The idea of preparing hydroxy amines directly from the amine by oxidation was also briefly explored. 2-nitrofluorene was used to test the activated arylamine method. The reduction was performed with hydrazine hydrate and palladium charcoal. The first experiments reacted N-trifluoroacetyl,N-acetoxy-2-aminofluorene with deoxyguanosine

monophosphate. A second method was also developed to react N-hydroxy-2-aminofluorene directly with deoxyguanosine monophosphate in an inert atmosphere, and in the presence of acetylsalicylic acid without previous isolation.

The traditional preparation of 2-nitroanthracene contains seven steps including a Friedel Crafts reaction, a nitration and several dehydrations,⁸¹ making it long and difficult. This scheme is presented in Figure 16.

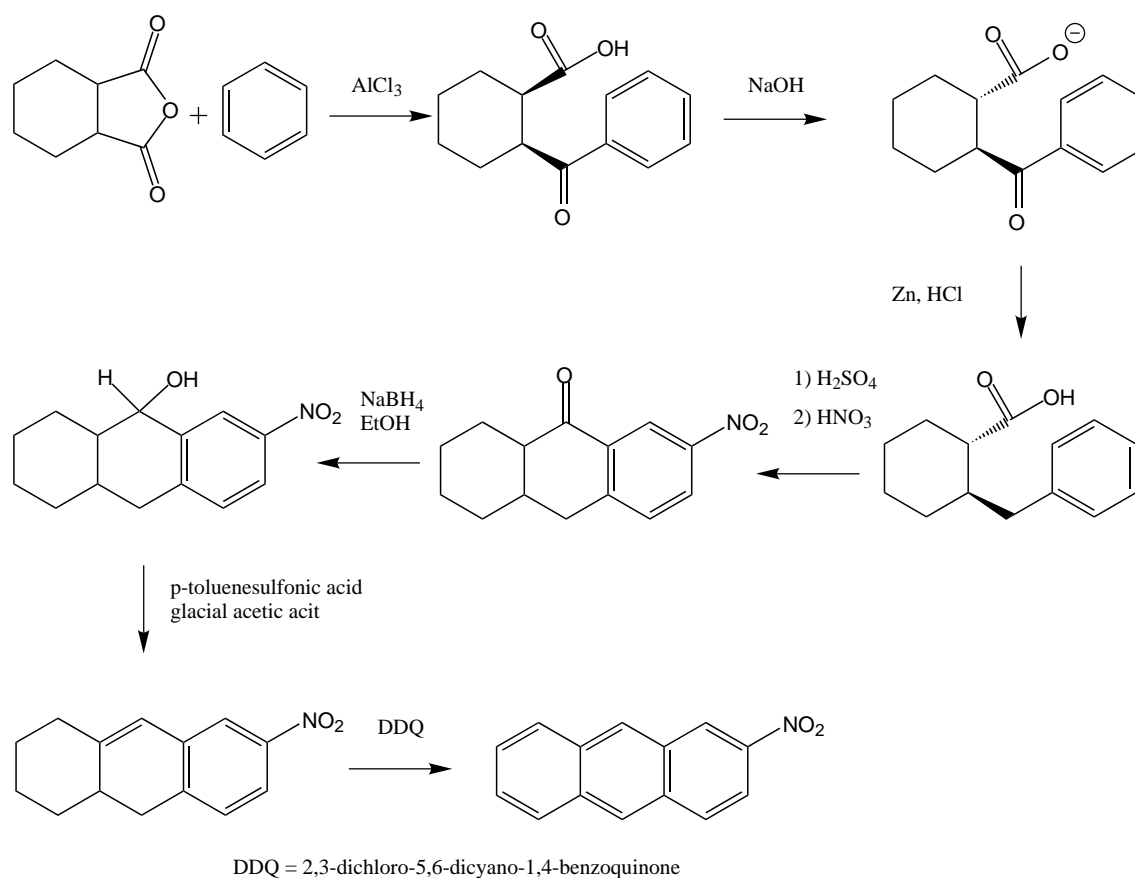


Figure 16. The synthesis of 2-nitroanthracene

In order to avoid this, a procedure was found to prepare 2-nitroanthracene by the six-electron oxidation of 2-AA with m-chloroperbenzoic acid.⁸² After flash chromatography 2-nitroanthracene was isolated in 56% yield. GC/MS of this compound showed the

correct mass and a unique retention time versus that of 9-nitroanthracene. Magnesium monoperoxyphthalate was also tried as an oxidant but found to offer no advantage.⁸³

If hydroxy amines could be prepared from their corresponding amines, it would exclude the need for nitro compounds. The literature was examined to see how likely this approach would be. One method reported excellent results for the synthesis of hydroxy amines from non-aromatic amino-saccharides and the methyl esters of amino acids, using the reagent 2,2-dimethyldioxirane.⁸⁴ This method was not tried however because 2,2-dimethyldioxirane is unstable and must be prepared *in situ* or immediately prior to use, and it has been known to completely oxidize arylamines to their nitro counterparts.⁸⁵ A different method was found that used benzoyl peroxide to create a benzoyl amine that would then give the hydroxylamine when treated with base.⁸⁶ Aniline was chosen as a model arylamine, but this method was abandoned after several attempts when it was determined that the benzyl amide was being produced instead of the benzoyl intermediate.

The reduction of 2-nitrofluorene to N-acetoxy-N-trifluoroacetyl-2-aminofluorene appeared to go smoothly. The reaction could be followed by TLC and a FAB mass spectrum was obtained of the product that did not contain a molecular ion, but did contain two likely fragments as the most abundant ions. These fragments were of 292 m/z from C-O cleavage of the acetoxy group, and 276 m/z from N-O cleavage of the acetoxy group. After incubation with deoxyguanosine monophosphate, and acid hydrolysis, direct HPLC/MS analysis revealed the presence of the non-acetylated C8-guanine 2-aminofluorene adduct in small amounts. Tandem mass spectrometry of the molecular ion produced the characteristic fragments seen in Figure 17.

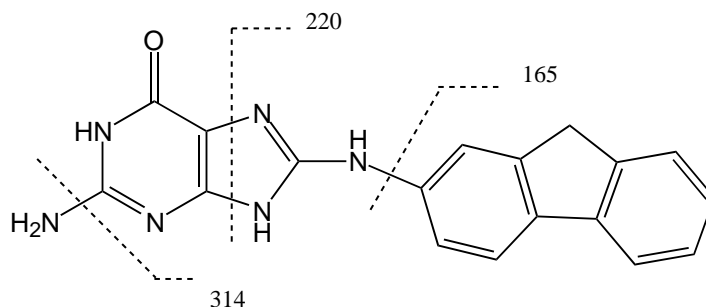


Figure 17. Fragments of the C8-G 2-aminofluorene adduct

These are the same fragments found by similar methods reported in the literature.⁶⁸ Attempts to isolate this compound by collecting fractions from an analytical column did not succeed. It is possible that not enough material was collected from subsequent runs in order to be detected, or that the wrong peaks were collected. Attempts to purify the reaction products by chromatography on silica also failed. The direct reaction of N-hydroxy-2-aminofluorene in the presence of acetylsalicylic acid had no noticeable affect on yield.

3.4 Alternate Adduct Synthetic Pathways

The two alternate types of adduct syntheses previously discussed were also attempted. The first tried was that of Swenson, that goes through a thiourea intermediate after reacting the arylamine with 6-hydroxy-2,4,5-triaminopyrimidine. The other method tried was the direct reaction of 2AA with 8-bromoguanosine-2',3',5'-triacetate. Neither method was successful, but recent attempts at the later could be worthwhile if a suitable HPLC purification method could be found.

Swenson's multistep method involves converting the arylamine, which is a poor nucleophile, into a good electrophile, and then reacting it with one of the amines present on a guanine analog. Suitable electrophiles are either the dithiocarbamate, or the isothiocyanate of the arylamine. When either of these compounds are reacted with 6-

hydroxy-2',4',5'-triaminopyrimidine they form a thiourea linked adduct. By removing the sulfur atom, and performing a ring closure on the intermediate, the molecule is converted to the C8 guanine adduct. Both methods were tried without success. The dithiocarbamate was prepared by reacting 2AA with CS₂ in 30% NH₄OH and precipitating. Matrix assisted laser desorption ionization MS did not detect a molecular ion but it might have fragmented before reaching the detector. Isothiocyanates of arylamines were prepared by the literature procedure using the reagent 1',1'-thiocarbonyldiimidazole.⁸⁷ This reaction is outlined in Figure 18.

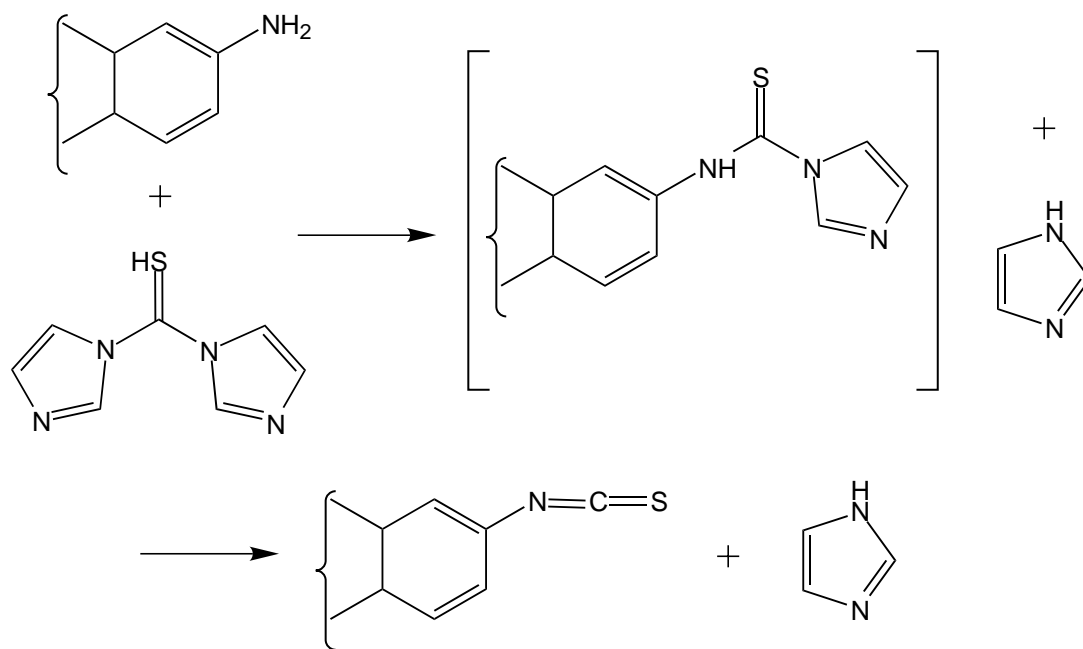


Figure 18. The isothiocyanate preparation of arylamines

The isothiocyanates of 2AA, 2-aminofluorene, and 2-aminonaphthalene were prepared in 82-90% yield and confirmed by GC/MS. Coupling of the dithiocarbamate of 2-AA or any of the isothiocyanates with 6-hydroxy-2,4,5-triaminopyrimidine were unsuccessful. NMR indicated more peaks than were expected for the product, and incomplete

conversion of starting material. FAB MS did not indicate product formation either. HPLC purification and analysis by ESI-MS were also unsuccessful.

The first attempt at coupling an arylamine with 8-bromoguanosine-2',3',5'-triacetate was based on the early procedure reported by Kriek et al³⁴. The components were dissolved in a small amount of isopropanol and sealed in glass tube. After heating the tube was opened and the solid inside was washed with methanol and then hydrolyzed in ethanol/1N HCl. This was then precipitated with additional HCl. This precipitate was purified by HPLC but no fractions were found to contain product by ESI-MS. No improvement was found by repeating the reaction at atmospheric pressure in DMSO. When it was discovered in the literature that this reaction is acid catalyzed, the hydrobromide of 2AA was prepared and the reaction was tried once more in refluxing isopropanol. Online HPLC/MS revealed the formation of completely hydrolyzed product in relatively large amounts. Preparative HPLC succeeded in slightly purifying this compound but was never optimized. Since depurination was intended for the adduct anyway, in this case cleavage of the glycosidic linkage was actually an advantage.

3.5 Adduct Synthesis with Palladium Catalysis

Recently the use of palladium catalysts for the formation of similar adducts indicated it might be possible to use them to make C8 adducts in high yield. After reviewing the literature for the Buchwald/Hartwig catalysis, a method for the coupling of a substituted 2-chlorobenzimidazole with a primary alkylamine was selected as a starting point for synthesis of C8-guanine adducts.⁸⁸ This scheme is outlined in Figure 19.

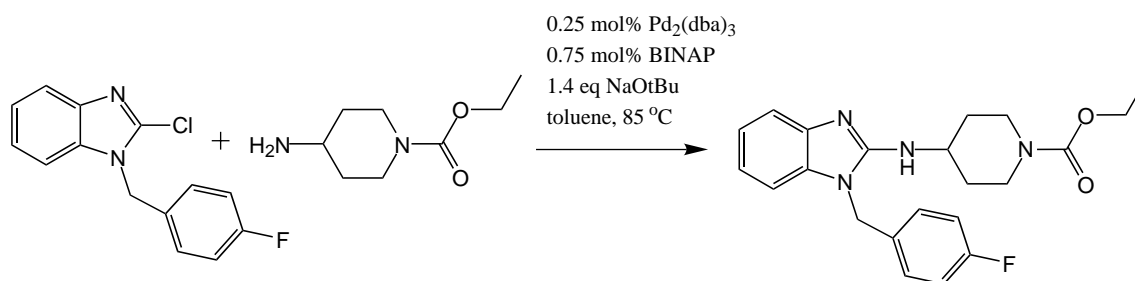


Figure 19. Palladium catalyzed amination of a chloroimidazole

It was decided to begin with the reaction of aniline and 8-bromo-adenosine as a simplified model for the desired reaction. This was because aniline is the simplest and smallest arylamine and adenosine offers improved solubility over guanosine and does not contain a reactive carbonyl functional group. The modified reaction we developed is outlined in Figure 20.

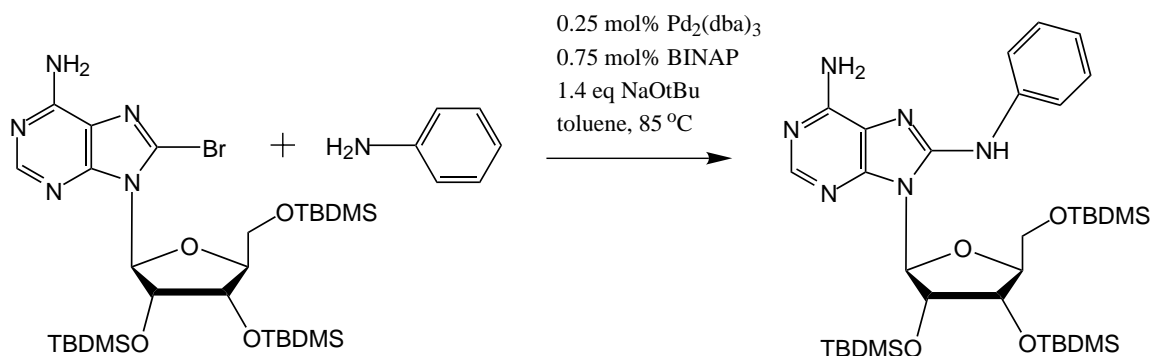


Figure 20. Coupling of protected adenosine and aniline

While 8-bromo-adenosine and 8-bromo-guanosine are both commercially available and relatively inexpensive for modified nucleosides, the reactivity of the hydroxyl groups on the purine ribose requires that they be protected. *Tert*-butyldimethylsilyl groups are ideal for this and are commonly used. 8-Bromo-adenosine is easily silylated and purified by the conventional method⁸⁹ used for unsubstituted bases,

but 8-bromoguanosine is slightly harder to purify due to its increased polarity. 8-Bromo-2',3',5'-tris-O-(*tert*-butyldimethylsilyl)-adenosine was prepared in 96.7% yield and 8-bromo-2',3',5'-tris-O-(*tert*-butyldimethylsilyl)-guanosine in 80.1%. The reaction proceeds rapidly with only trace amounts of mono- and di-protected species forming. These minor impurities are easily separated by flash chromatography. Due to steric hinderence, each of the six silyl methyls has a unique ^1H NMR frequency, as do the three *t*-butyl groups. This is seen in Figure 21.

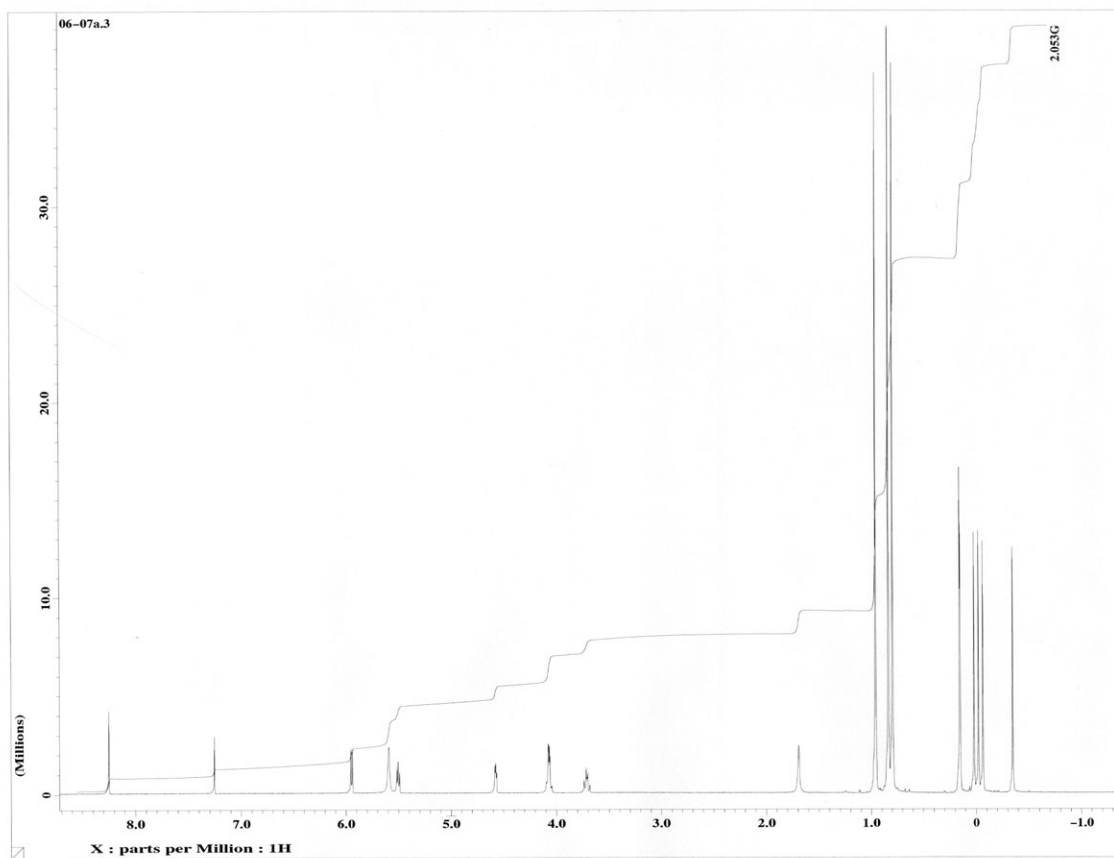


Figure 21. ^1H NMR spectrum of protected 8-bromo-adenosine

The coupling with aniline proceeded smoothly, giving product in 84% yield. ^1H NMR of this adduct indicates success by the relative integration of the aromatic protons

and reasonable shifting of the amine peaks. The ribose protons are also shifted and split in a characteristic fashion found in all other adducts made. This can be seen in Figure 22.

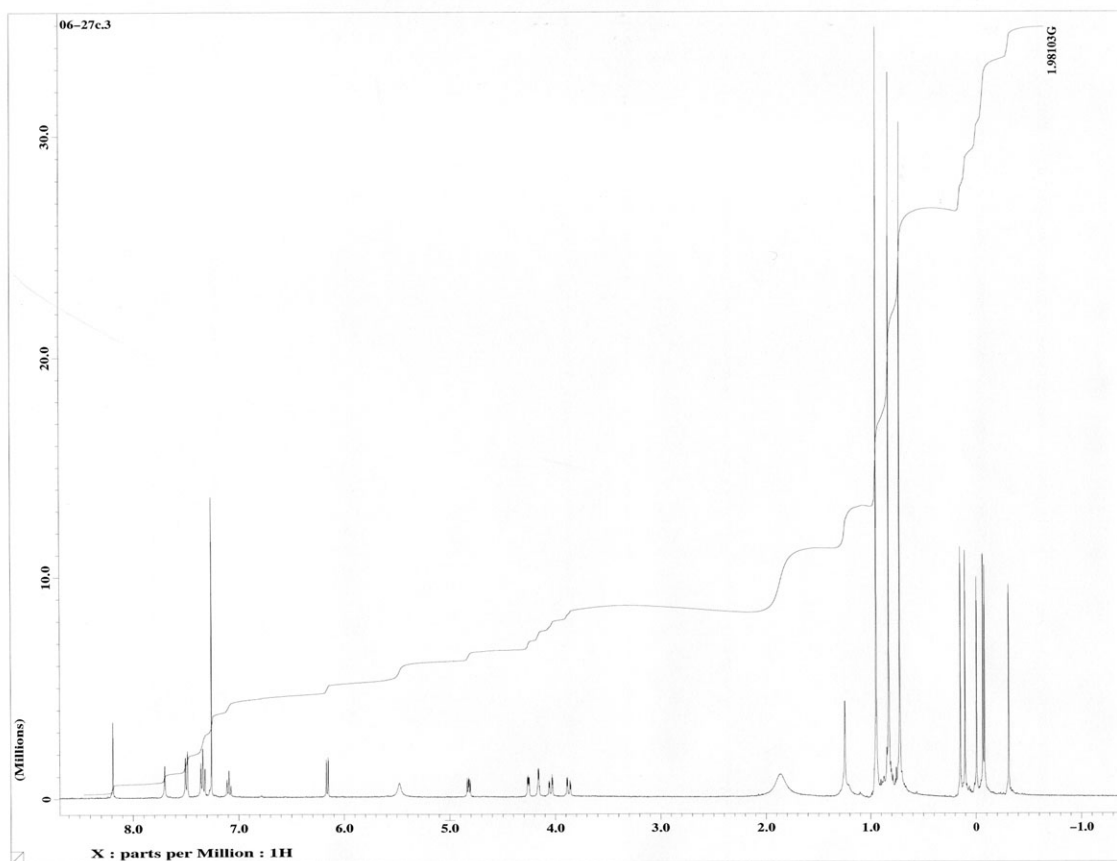


Figure 22. ^1H NMR spectrum of the aniline-adenosine adduct

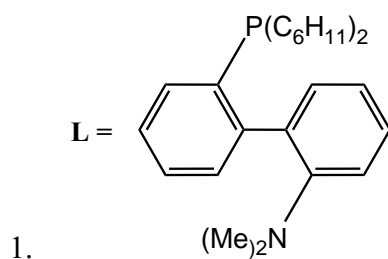
Unfortunately, coupling with the larger arylamines, 2-aminoanthracene and 2-aminofluorene, were low yielding and difficult to purify. Different reaction conditions were tried, using alternate ligands, bases, and solvents to improve yield, while different

solvent mixtures were tried to improve isolation by flash chromatography on silica gel. Aside from BINAP two additional ligands were tried. The ligand 2-dicyclohexylphosphino-2'-(N,N-dimethylamino)biphenyl was used by Lakshman for his N6-aryl-adenosine adducts, and the other ligand, 2-(Di-*t*-butylphosphino)biphenyl, was developed by the Buchwald group to improve the coupling of primary anilines with electron rich aryl bromides. Both ligands are recently available from Strem Chemicals. The results of several reactions for the coupling of 2-aminofluorene with protected 8-bromo-adenosine are presented in Table 3.

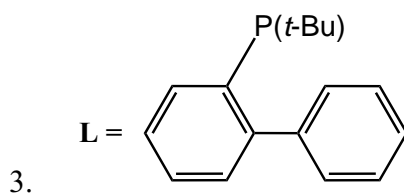
The biphenyl ligands did not perform as well as BINAP and choice of base seemed to have no effect. The conditions of Lakshman were designed to improve the solubility of the mild base K_3PO_4 , but they were found ineffective and no noticeable degradation of the nucleoside was found to occur from the harsher base $NaOtBu$. 3:1 Hexane/ethyl acetate was sufficient for chromatography of the aniline adduct, but it did not work for any of the others. 5% acetone/dichloromethane, used by Lakshman, also did not work but 5% methanol/chloroform was successful in isolating the benzidine adduct in 60% yield. This mixture was too polar to isolate the 2-aminofluorene, and 4-aminobiphenyl adducts, but 0.5% methanol/chloroform was used to purify them in 28%, and 26% respective yield. It is possible that the low polarity of this solution prevents recovery of the entire amount of compound from the silica, but it is required for the sake of purity. This problem is exacerbated by the high catalytic loading (30 mol % Pd, 50 mol % BINAP) that we found was needed for complete loss of starting material. The coupling of 2AA was even poorer yielding and a suitable procedure for isolation was not found, although HPLC/MS of the crude reaction indicated some product was formed.

Table 3. Reaction Coupling Conditions

Solvent	Pd ₂ (dba) ₃	Ligand	Base	Results
1,2-Dimethoxyethane	10 mol %	30 mol % L ¹	K ₃ PO ₄	No Reaction ²
Toluene	10 mol %	30 mol % L ¹	Cs ₂ CO ₃	<30 % Crude Yield
Toluene	10 mol %	30 mol % L ³	NaOtBu	No Reaction
Toluene	10 mol %	30 mol % BINAP	K ₃ PO ₄	<30 % Crude Yield
Toluene	10 mol %	30 mol % BINAP	Cs ₂ CO ₃	<30 % Crude Yield
Toluene	10 mol %	30 mol % BINAP	NaOtBu	<30 % Crude Yield



2. Conditions used by Lakshman⁵⁵



When palladium acetate ($\text{Pd}(\text{OAc})_2$) was used as a source of palladium instead of tris-dibenzylideneacetone-di palladium ($\text{Pd}_2(\text{dba})_3$) faster reaction times were observed but yields did not improve. Typical reactions with $\text{Pd}(\text{OAc})_2$ showed a complete loss of starting material after 6 hours, while those with $\text{Pd}_2(\text{dba})_3$ took 8-24 hours. $\text{Pd}(\text{OAc})_2$ did require premixing with the ligand before addition of the base, because base was found to prevent formation of the active catalyst.⁵⁴ To determine if the chirality of BINAP had any effect on coupling, the reaction of aniline and protected adenosine was tried separately with either R or S-BINAP as opposed to the usual racemic mixture. Yield was determined by HPLC and was found to be 77% for R-BINAP and 87% for S-BINAP, indicating that chirality had little or no effect. A possible explanation for this is that the C-N bond that is formed is not chiral itself, or that the sterically hindering chiral parts of the BINAP and the nucleoside are too far away from the active site of the catalyst to control bond formation.

Attempts to couple 2-chloroaniline, 4-chloroaniline, or 3,3'-dichlorobenzidine with protected adenosine were mostly unsuccessful although some crude NMR spectra indicated that product might be forming in low amounts. These compounds all have halides attached to them however, and this offers a site for self-polymerization, where the amine couples at a chlorine position on a similar molecule, rather than at the bromine position on a nucleoside molecule. 8-Bromo-adenosine also has an amine that allows for this type of side reaction, but it has been found that the reactivity of the amine is key for this reaction to proceed.⁹⁰ Johnson has demonstrated that these types of dimers or even trimers are possible to create but that the N2 of guanosine is much more reactive than the N6 of adenosine. This is illustrated in Figure 23.

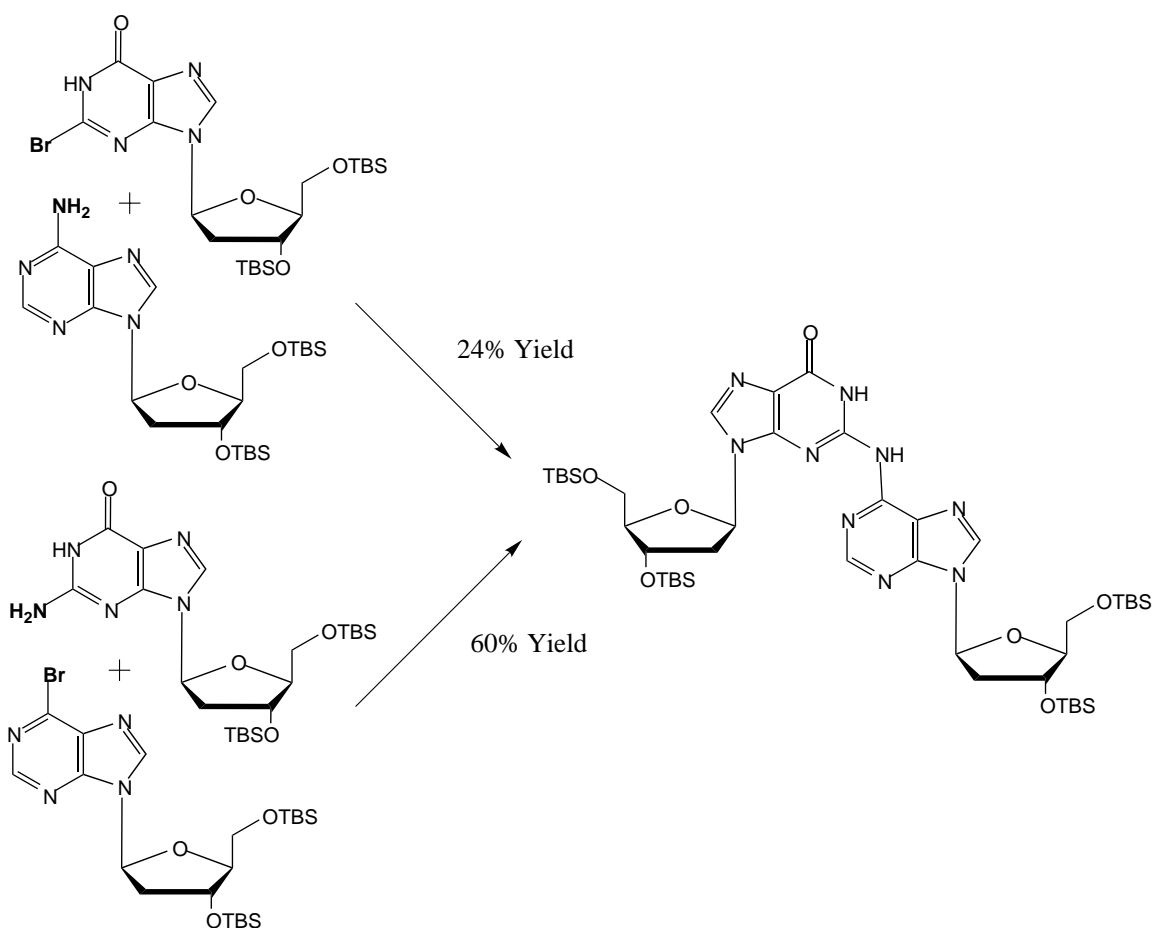


Figure 23. Nucleoside to nucleoside coupling

In spite of these results no adenosine dimer or polymer was detected and protection of the adenosine amine was not required. Alternately, 8-bromo-2',3',5'-tris-O-(*t*-butyldimethylsilyl)-guanosine would not couple with any arylamine most likely due to interference from its amine and carbonyl functionalities interacting with the catalyst.

Overall, four arylamine adenosine adducts were prepared. These compounds were characterized by ^1H and ^{13}C NMR, IR, high-resolution mass spectrometry, and HPLC/ESI mass spectrometry. The deuterated versions of two of these arylamines were also prepared. The yields of these reactions are summarized in Table 4.

Table 4. Arylamine-Adenosine Adduct Yield

Arylamine	% Yield
Aniline	84
Benzidine	60
4-Aminobiphenyl	28
2-Aminofluorene	25
d₈ Benzidine	28
d₉ 2-aminofluorene	26

Although these yields are not exceptional they are much higher than those reported for nitrenium ion methods, and they allow for the easy preparation of large amounts of material for use or NMR analysis. The authentic and deuterated benzidine and 2-aminofluorene adducts were hydrolyzed with HCl and used as standards for a quantitative HPLC tandem mass spectrometry detection method.

The adducts were hydrolyzed by dissolving in ethanol, adding an equal amount of 1 N HCl and refluxing for 1 hour. The ethanol was then removed under reduced pressure and the solution, along with any precipitate, was loaded onto a C-18 Sep-Pak cartridge. It was then washed with water and eluted with two 5ml portions of acetonitrile and one of methanol. This elution worked for the benzidine adduct which came off in the methanol, but the 2-aminofluorene adduct came off in the first acetonitrile fraction and was impure. This was overcome by eluting with 20%, 40%, 60%, 80% and 100% mixtures of

acetonitrile in water. The first three fractions contained the 2-aminofluorene adduct free from impurities. These adducts were then used to develop an analytical method.

3.6 Experimental

Dry DMF and toluene were purchased from ACROS in septum capped bottles and used as such. All other solvents used were ACS grade or higher. All reactions were performed under an atmosphere of argon. R-, S-, and racemic BINAP, 2-dicyclohexylphosphino-2'-(N,N-dimethyl-amino)biphenyl, and 2-(di-*t*-butylphosphino)biphenyl were purchased from Strem, Newburyport MA and used as received. All other compounds were purchased from Aldrich and used without further purification except for 2-aminofluorene that was recrystallized once from 50% aqueous ethanol. Analytical thin layer chromatography was performed using Alltech pre-coated silica gel UV/254 plastic plates. Compounds were visualized by dipping the plates in a KMnO_4 solution and then rinsing with DI water. Flash column chromatography was performed using the indicated solvent on Scientific Adsorbents Inc. silica gel (32-63 mm, Atlanta GA). IR spectra were recorded in dichloromethane on salt plates using a Bruker Equinox 55 spectrometer. ^1H and ^{13}C NMR were recorded in CDCl_3 using a JEOL Eclipse 400 FT NMR spectrometer. HRMS was performed by Pharmacia.

2-Aminoanthracene- d_7

A 50 mL Schlink flask was flushed with argon and charged with 100 mg of Pt^0 and 10 mL D_2O . 200 mg of 2-aminoanthracene dissolved in 15 mL of EtOD was then added and the mixture was refluxed for 48 hours under argon. After cooling the mixture was filtered and washed with 3 x 10 mL of DCM. The organic phase was then washed

with 3 x 20 mL of water and the concentrated under vacuum. The reaction was repeated four more time with fresh Pt⁰ and D₂O. The final product was then analyzed by GC/MS.

2-Nitrofluorene-d₉

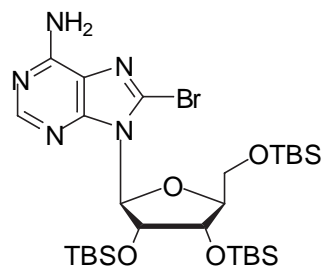
Prepared by the literature route using fluorene-d₁₀. (Khun, W. E. *Organic Syntheses*, Coll. Vol. 2, **1933**, 447) Analyzed by GC/MS.

2-Aminofluorene-d₉

Prepared by the literature route using 2-nitrofluorene-d₉. (Bavin, P. M. *Organic Synthesis*, Vol. 40, **1960**, 5.) Analyzed by GC/MS.

2-Nitroanthracene

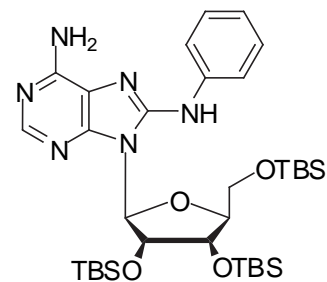
Oxidized form 2-aminoanthracene by the literature procedure using m-chloroperbenzoic acid. (Scribner, J. D.; Fisk, S. R.; Scribner, N. K. *Chem-Biol. Interactions* **1979**, 26, 11.) Analyzed by GC/MS.



8-bromo, 2',3',5'-tris-O-(*tert*-butyldimethylsilyl)-adenosine

Prepared by a modified literature procedure.(Ogilvie, K. K.; Beaucage, S. L.; Scifman, A. L.; Theriault, N. Y.; Sadana, K. L. *Can. J. Chem.* **1978**, 56, 2768-2780) For a typical reaction, an oven dried Schlenk flask that was cooled under vacuum, flushed

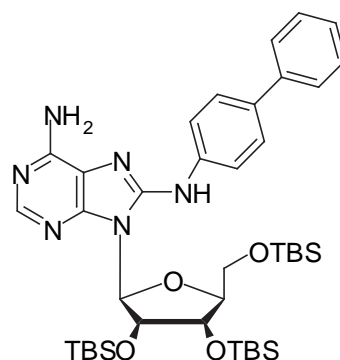
with Argon, and charged with 8-bromo-adenosine (300 mg, 0.87 mmol) and imidazole (473 mg, 6.96 mmol). Dry DMF (5 mL) was added via syringe followed by tert-butyltrimethylchlorosilane (525 mg, 3.48 mmol). The system was flushed once more with Argon and allowed to stir at room temperature for 24 h. TLC showed one major spot (rf 0.65) followed by two minor ones (rf 0.51, 0.46). (10 ml) Water was then added and the product was extracted with CHCl_3 (3 x 30 mL). The combined organic layers were washed once with saturated NH_4Cl , twice with brine, and then dried over MgSO_4 . The drying agent was removed by filtration and the solvent was evaporated to leave a white powder. The crude material was purified by flash chromatography (3:1 hexane/EtOAc) affording first the title compound (512 mg, 86%) and a mixture of the mono and di protected nucleoside (64 mg, 11%). IR 3331, 3187, 2928, 2856, 1668, 1604, 1576, 1458, 1146, 1055, 779, 335 cm^{-1} ; ^1H NMR (CDCl_3) δ 8.25 (s, 1 H), 5.94 (d, J = 5.5 Hz, 1 H), 5.55 (bs, 2 H), 5.50 (dd, J = 4.4, 5.8 Hz, 1 H), 4.57 (dd, J = 2.2, 4.4 Hz, 1 H), 4.07 (m, 2 H), 3.71 (m, 1 H), 0.96 (s, 9 H), 0.83 (s, 9 H), 0.79 (s, 9 H), 0.16 (s, 3 H), 0.15 (s, 3 H), 0.02 (s, 3 H), -0.02 (s, 3 H), -0.07 (s, 3 H), -0.35 (s, 3 H); ^{13}C NMR δ 154.2, 152.7, 151.0, 129.0, 120.6, 90.7, 85.7, 72.3, 71.8, 62.3, 26.0, 25.9, 25.8, 18.4, 18.2, 17.9, -4.4, -4.5, -5.1, -5.3, -5.5. Elemental analysis for $\text{C}_{28}\text{H}_{54}\text{BrN}_5\text{O}_4\text{Si}_3$ calculated 48.82% C, 7.90% H, 10.17% N, found 49.01% C, 7.77% H, 10.22% N. HRMS calculated for ($\text{M}^+ + 1$): 688.2745, found: 688.2757.



N-(2',3',5'-tris-O-*tert*-butyldimethylsilyladenodin-8-yl)-aniline

Prepared by a modified literature procedure (Hong, Y.; Tanoury, G. J.; Wilkinson, H. S.; Bakale, R. P.; Wald, S. A.; Senanayake, C. H. *Tetrahedron Letters*, **1997**, 38, 5607-5610. For a typical reaction, an oven dried Schlenk flask and condenser was cooled under vacuum, flushed with Argon, and charged with 8-bromo-2',3',5'-tris-O-(*tert*-butyldimethylsilyl)-adenosine (20 mg, 0.03 mmol), Pd₂(dba)₃ (0.4 mg, 1.25 mol%), BINAP (0.7 mg, 3.75 mol%), aniline (3.6 mL, 0.039 mmol), NaOtBu (4.04 mg, 0.042 mmol), and anhydrous toluene (5 mL). The temperature of the orange solution was brought to 80 °C using an electronically controlled oil bath. After 6 h all starting material was consumed (TLC). The solution was transferred to a round bottom flask and the solvent was evaporated. The crude mixture was purified by flash column chromatography (gradient 3:1 hexane/EtOAc, to 1:1 hexane/EtOAc, rf 0.08) which afforded the title compound as a purple solid (17.6 mg, 84%). IR 3353, 3160, 2957, 2929, 2858, 1662, 1639, 1599, 1556, 1470, 1255, 1165, 1103, 829, 774 cm⁻¹; ¹H NMR d 8.19 (s, 1 H), 7.70 (s, 1 H), 7.49 (bd, J = 7.7 Hz, 2 H), 7.34 (dd, J = 7.7, 7.7 Hz, 2 H), 7.09 (dd, J = 7.7, 7.7 Hz, 1 H), 6.16 (d, J = 7.0 Hz, 1 H), 5.48 (br, 2 H), 4.82 (dd, J = 4.6, 7.1 Hz, 1H), 4.26 (dd, J = 2.2, 4.8 Hz, 1 H), 4.16 (bd, J = 2.2 Hz, 1 H), 4.05 (dABq, J = 2.2, 11.7 Hz, 1 H), 3.87 (dABq, J = 2.6, 11.7 Hz, 1 H), 0.95 (s, 9 H), 0.83 (s, 9 H), 0.72

(s, 9 H), 0.15 (s, 3 H), 0.10 (s, 3 H), -0.01 (s, 3 H), -0.07 (s, 3 H), -0.08 (s, 3 H), -0.31 (s, 3 H); ^{13}C NMR d 152.4, 150.3, 148.7, 139.0, 129.0, 123.4, 120.8, 117.3, 86.5, 86.4, 72.5, 72.1, 63.1, 29.8, 26.2, 26.0, 25.6, 18.8, 18.2, 17.8, -4.3, -4.5, -4.6, -5.1, -5.2, -5.4. HRMS calculated for ($\text{M}^+ + 1$): 701.4062, found: 701.4056.

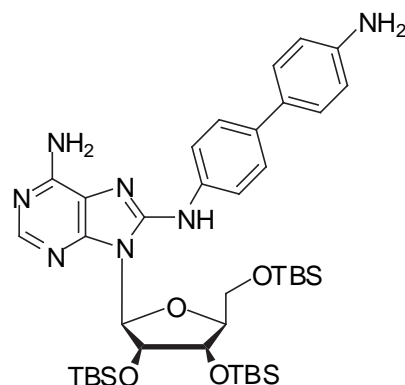


N-(2',3',5'-tris-O-*tert*-butyldimethylsilyladenodin-8-yl)-4-aminobiphenyl

Prepared by the same procedure as the N-(2',3',5'-tris-O-*tert*-butyldimethylsilyladenodin-8-yl)-aniline, using 8-bromo-2',3',5'-tris-O-(*tert*-butyldimethylsilyl)-adenosine (40 mg, 0.06 mmol), and 4-aminobiphenyl (30.4 mg, 0.18 mmol). 30 Mol% $\text{Pd}_2(\text{dba})_3$, and 50 Mol% BINAP were used. The product was purified by flash chromatography (1% MeOH / CHCl_3 , rf 0.21) and isolated as a brown solid (27.5 mg, 56%). IR 3354, 2929, 2863, 1631, 1590, 1556, 1464, 1256, 1171, 1105, 836, 778 cm^{-1} ; ^1H NMR d 8.21 (s, 1 H), 7.77 (s, 1 H), 7.58 (m, 6 H), 7.44 (m, 2 H), 7.33 (m, 1 H), 6.17 (d, $J = 7.0$ Hz, 1 H), 5.36 (br, 2 H), 4.85 (dd, $J = 4.8, 7.0$ Hz, 1 H), 4.28 (dd 1 H, $J = 2.2, 4.8$ Hz, 1 H), 4.18 (d, $J = 2.6$ Hz, 1 H), 4.07 (dABq, $J = 2.2, 11.7$ Hz, 1 H), 3.89 (dABq, $J = 2.8, 11.9$ Hz, 1 H), 0.95 (s, 9 H), 0.85 (s, 9 H), 0.73 (s, 9 H), 0.16 (s, 3 H), 0.11 (s, 3 H), 0.02 (s, 3 H), -0.05 (s, 3 H), -0.05 (s, 3 H), -0.29 (s, 3 H); ^{13}C NMR

d 152.5, 150.2, 150.2, 148.6, 140.9, 138.2, 136.0, 129.0, 127.7, 127.0, 126.9, 120.9, 86.5, 86.4, 72.7, 72.1, 63.2, 26.3, 26.0, 25.6, 18.8, 18.25, 17.8, -4.4, -4.6, -5.0, -5.2, -5.4.

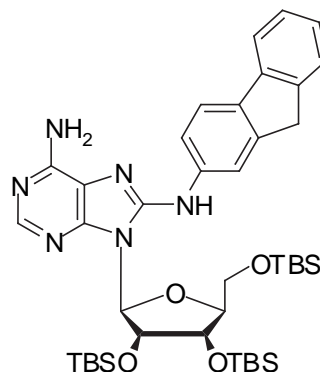
HRMS calculated for ($M^+ + 1$): 777.4375, found: 777.4362.



N-(2',3',5'-tris-O-*tert*-butyldimethylsilyladenoin-8-yl)-benzidine

Prepared by the same procedure as N-(2',3',5'-tris-O-*tert*-butyldimethylsilyladenoin-8-yl)-aniline, using 8-bromo-2',3',5'-tris-O-(*tert*-butyldimethylsilyl)-adenosine (40 mg, 0.06 mmol), and benzidine (33 mg, 0.18 mmol). 30 Mol% $\text{Pd}_2(\text{dba})_3$, and 50 Mol% BINAP were used. Purification was by flash chromatography (1% MeOH / CHCl_3 to 5% MeOH / CHCl_3 , rf 0.40 5% MeOH / CHCl_3) as a brown solid (28.7 mg, 60%). IR 3453, 3354, 3219, 3031, 2959, 2935, 2851, 1637, 1602, 1555, 1497, 1467, 1253, 1156, 1106, 837, 776, 737; ^1H NMR d 8.21 (s, 1 H), 7.68 (s, 1 H), 7.51 (dd, $J = 8.6, 16.7$ Hz, 4 H), 7.39 (d, $J = 8.4$ Hz, 2 H), 6.75 (d, $J = 11.0$ Hz, 2 H), 6.16 (d, $J = 7.0$ Hz, 1 H), 5.25 (br, 2 H), 4.86 (dd, $J = 4.8, 7.0$ Hz, 1 H), 4.28 (dd, $J = 2.4, 4.6$ Hz, 1 H), 4.17 (d, $J = 2.6$ Hz, 1 H), 4.05 (dABq, $J = 2.4, 11.9$ Hz, 1 H), 3.88 (dABq, $J = 2.6, 11.7$ Hz, 1 H), 0.95 (s, 9 H), 0.84 (s, 9 H), 0.73 (s, 9 H), 0.15 (s, 3 H), 0.11 (s, 3 H), 0.01 (s, 3 H), -0.05 (s, 3 H), -0.06 (s, 3 H), -0.30 (s, 3 H); ^{13}C NMR d 152.4, 150.3, 150.2, 148.9, 145.7, 137.5, 136.5, 131.4, 127.7, 126.8, 120.9, 117.2,

115.5, 86.4, 86.3, 72.4, 72.2, 63.1, 26.3, 26.0, 25.7, 18.8, 18.2, 17.8, -4.2, -4.4, -4.6, -5.1, -5.2, -5.5. HRMS calculated for ($M^+ + 1$): 792.4484, found: 792.4470.



N-(2',3',5'-tris-O-*tert*-butyldimethylsilyladenodin-8-yl)-2-aminofluorene

Prepared by a modified procedure (Wolf, J. P.; Buchwald, S. L. *J. Org. Chem.* **2000**, *65*, 1144-1157.). An oven dried Schlenk flask was purged with argon. A solution of Pd(OAc)₂ (4.0mg, 30 mol%) and BINAP (18.6mg, 50 mol%) in 5mL anhydrous toluene was added and heated at 40 °C for 30 minutes. After the BINAP completely dissolved 2-aminofluorene (32.6mg, 0.18 mmol), 8-bromo-2',3',5'-tris-O-(*tert*-butyldimethylsilyl)-adenosine (40mg, 0.06 mmol), and NaOtBu (8.0mg, 0.084 mmol) was then added and the temperature was raised to 80 °C. After 5.5 hours the reaction was complete by TLC and the reaction mixture concentrated. The crude material was purified by flash column chromatography (eluted 0.5% MeOH in CHCl₃) and isolated as a greenish solid (12.0 mg, 25%). IR 3354, 2938, 2928, 2856, 1635, 1559, 1458, 1257, 1106, 836, 778; ¹H NMR d 8.20 (s, 1 H), 7.83 (s, 1 H), 7.74 (m, 3 H), 7.53 (bd, J = 7.6 Hz, 1 H), 7.44 (dd, J = 2.0, 8.2 Hz, 1 H), 7.37 (dd, J = 7.1, 7.1 Hz, 1 H), 7.29 (dd, J = 1.1, 7.3 Hz, 1 H), 6.20 (d, J = 7.7 Hz, 1 H), 5.61 (br, 2 H), 4.81 (dd, J = 4.8, 7.3 Hz, 1 H), 4.26 (dd, J = 2.2, 4.8 Hz, 1 H), 4.18 (d, J = 2.2 Hz, 1 H), 4.08 (dABq, J = 2.6, 11.7 Hz, 1

H), 3.90 (m, 3 H), 0.96 (s, 9 H), 0.83 (s, 9 H), 0.73 (s, 9 H), 0.15 (s, 3 H), 0.11 (s, 3 H), 0.01 (s, 3 H), -0.05 (s, 3 H), -0.07 (s, 3 H), -0.29 (s, 3 H); ^{13}C NMR d 151.5, 150.2, 149.4, 148.6, 144.4, 143.1, 141.5, 137.8, 137.5, 126.9, 126.3, 125.1, 120.6, 120.2, 119.6, 118.2, 117.1, 86.7, 86.3, 72.4, 72.3, 63.3, 37.1, 29.8, 26.3, 25.9, 25.6, 18.9, 18.2, 17.8, -4.3, -4.4, -4.5, -5.1, -5.2, -5.4. HRMS calculated for ($\text{M}^+ + 1$): 789.4375, found 789.4377.

CHAPTER 4. ADDUCT ANALYSIS BY HPLC TANDEM MASS SPECTROMETRY

4.1 Introduction and Objective

The detection of DNA adducts is a daunting challenge. Their formation is rare and the isolation of DNA as a sample matrix dictates that most samples will be small. Yet because carcinogens have no threshold of safety for exposure, no observable level of adverse effect, and exposure to a single molecule or “one hit” may be enough to cause uncontrollable cell division that is potentially fatal, it is important to detect them. Not only do they need to be detected, but also their formation and repair needs to be studied, and the complete mechanism of their carcinogenicity understood, in hopes that something can be done to prevent their ill effects.

The detection of DNA adducts by HPLC electrospray ionization (ESI) tandem mass spectrometry (MS/MS) offers several advantages that other methods do not possess. This method offers the advantages of not requiring radioactive compounds, or hard to obtain antibodies. It also provides specific qualitative information in the form of molecular weights and characteristic fragmentation. Aside from serving as a useful tool to study adduct formation and the mechanisms of tumor initiation, this method also measures direct evidence of primary damage to DNA that measuring the tissue concentration of the parent arylamines does not do. Measuring DNA adducts themselves is thought to be a more precise and reliable way to determine the risks from carcinogen exposure.^{91,92} Consequently the use of DNA adducts as bio-markers for risk assessment and environmental monitoring is increasing.⁹³

We have developed a qualitative and quantitative HPLC, ESI, MS/MS, isotope dilution detection method to detect the C8 adenine adducts of benzidine and 2-

aminofluorene. In order to demonstrate the validity of this method two separate spike and recovery studies were performed.

4.2 Experimental Section

C8-Adenine adducts of per-deutero, and non-labeled 2-aminofluorene and benzidine were prepared using our new method of palladium catalysis. C-18 Sep-Pak cartridges were purchased from Waters (Milford, MA). All solvents used for LC/MS and LC/MS/MS analysis were HPLC grade and purchased from Fisher Scientific (Itasca, IL). All mobile phases contained 0.2% formic acid to assist in ESI positive ionization. Separation was achieved using a Waters 2696 multisolvent delivery system equipped with a Waters 2487 dual wavelength UV detector and an auto-injector. Reverse-phase chromatography was performed using a Zorbax SB-CN column (250 x 4.6 mm, 5 μ m) purchased from MAC-MOD Analytical, Inc. (Chadds Ford, PA). The flow rate used was 1 mL/min. with a 1:1 split between the column and mass spectrometer. 0.2% formic acid in water was used as solvent A, and 0.2% formic acid in acetonitrile was used as solvent B with the following linear gradient program. 0 minutes, 100% A; 5 minutes, 100% A; 25 minutes, 100% B; 30 minutes 100% B; 35 minutes 100% A; 40 minutes 100% A.

Mass spectrometry was performed with a Micromass (Wythenshawe, UK) Quatro II triple-quadrupole mass spectrometer using the electrospray ionization source in the positive ion mode. Tuning of the ionization source and optimization of the MS/MS parameters were carried out using non-labeled C8-adenine adducts of 2-aminofluorene and benzidine. Roughly 5ng/ μ L standards were prepared in methanol containing 0.2% formic acid and infused into the mass spectrometer with a syringe pump at a flow rate 10 μ L/min. After the ESI interface was optimized and a general tune was performed on

each adduct the optimum ionization voltage for each adduct was determined. This was done by monitoring the intensity of the $[M+H]^+$ ion (m/z 318, benzidine adduct, m/z 315, 2-aminofluorene adduct) over a 2.0 kV to 5.0 kV range of capillary voltages in 0.2 kV increments. The collision energy was then optimized by monitoring the intensity of the most abundant fragment ion produced from each parent ion. In this study the first quadrupole of the mass spectrometer was set to pass only the parent ion, collisions were induced in the central region with a gas cell pressure of $\sim 2.5 \times 10^{-3}$ mbar argon and a full scan of fragment ions was acquired by the third quadrupole. Fragmentation of the benzidine adduct was monitored over collision voltages from 0 to 125 V, and fragmentation of the 2-aminofluorene adduct was monitored over voltages from 0 to 155 V as it required a higher fragmentation energy.

For the quantitative study of adducts a multiple reaction monitoring (MRM) method was developed for each adduct and its labeled internal standard. This involved passing only the molecular ion of the analytes (parent ions) through the first quadrupole, fragmenting in the collision cell, and then passing only the two most abundant fragments of each analyte (daughter ions) on to the detector. For the benzidine adduct the transitions monitored were m/z 318 to 169 and m/z 318 to 208, for its labeled counterpart they were m/z 326 to 175, and m/z 326 to 215. For the 2-aminofluorene adduct the transitions monitored were m/z 315 to 166, and m/z 315 to 205, for its labeled counterpart they were m/z 324 to 173, and m/z 324 to 205. A standard curve was constructed by replicate analysis of 10 μ L injections of standard solutions containing from 0.1 ng/ μ L, 0.2 ng/ μ L, 0.4 ng/ μ L, 0.8 ng/ μ L, 1.6 ng/ μ L, and 3.2 ng/ μ L of adduct in

DMSO. Each injection also contained 2 ng of the corresponding deuterated internal standard. Blanks both with and without internal standard were also analyzed.

Two spike and recovery studies were performed to assess method validity, adduct stability, and procedure efficiency. For the first study two sets of duplicate samples were prepared by spiking 15, 75, 150, and 300 ng of authentic standards into separate test tubes containing 2.5 mL of 0.02 N HCl. The solutions were then hydrolyzed for 1.5 hours at 75 °C. After cooling, each sample was neutralized to pH 7 with NaOH. Each sample was then loaded on to a C18 Sep-Pak cartridge that had been activated by rinsing with 6 mL of acetonitrile followed by 6 mL of DI water. After loading each sample was washed with 8 mL of DI water and then eluted with 3 mL of methanol. Each methanol sample was then evaporated to dryness under ultra pure nitrogen and then spiked with 30 ng of the appropriate internal standard dissolved in 50 µL of DMSO. The residue was then redissolved in 100 µL of DMSO and submitted to LC/MS/MS analysis. For the second spike and recovery study, two sets of duplicate samples were prepared by spiking 15, 75, 150, and 300 ng of authentic standards into separate test tubes containing 2.5 mL of 0.02 N HCl and 4mg of salmon sperm DNA. Each sample was then spiked with 30 ng of the appropriate internal standard at this point rather than in the post Sep-Pak stage. The solutions were then hydrolyzed for 1.0 hours at 75 °C. After cooling, each sample was neutralized to pH 7 with NaOH. Each sample was then loaded on to a C18 Sep-Pak cartridge that had been activated by rinsing with 6 mL of acetonitrile followed by 6 mL of DI water. After loading each sample was washed with 8 mL of DI water and then eluted with 3 mL of methanol. Each methanol sample was then evaporated to dryness

under ultra pure nitrogen. The residue was then redissolved in 150 μ L of DMSO and submitted to LC/MS/MS analysis.

4.3 Results and Discussion

4.3.1 Standard Characterization and Ionization Optimization

To insure the purity of each standard a full scan of each adduct was taken over a mass range of m/z 110 to 1000 by direct infusion. The ESI mass spectrum for the benzidine adduct, and that of its deuterated form are seen in Figures 24. and 25. respectively.

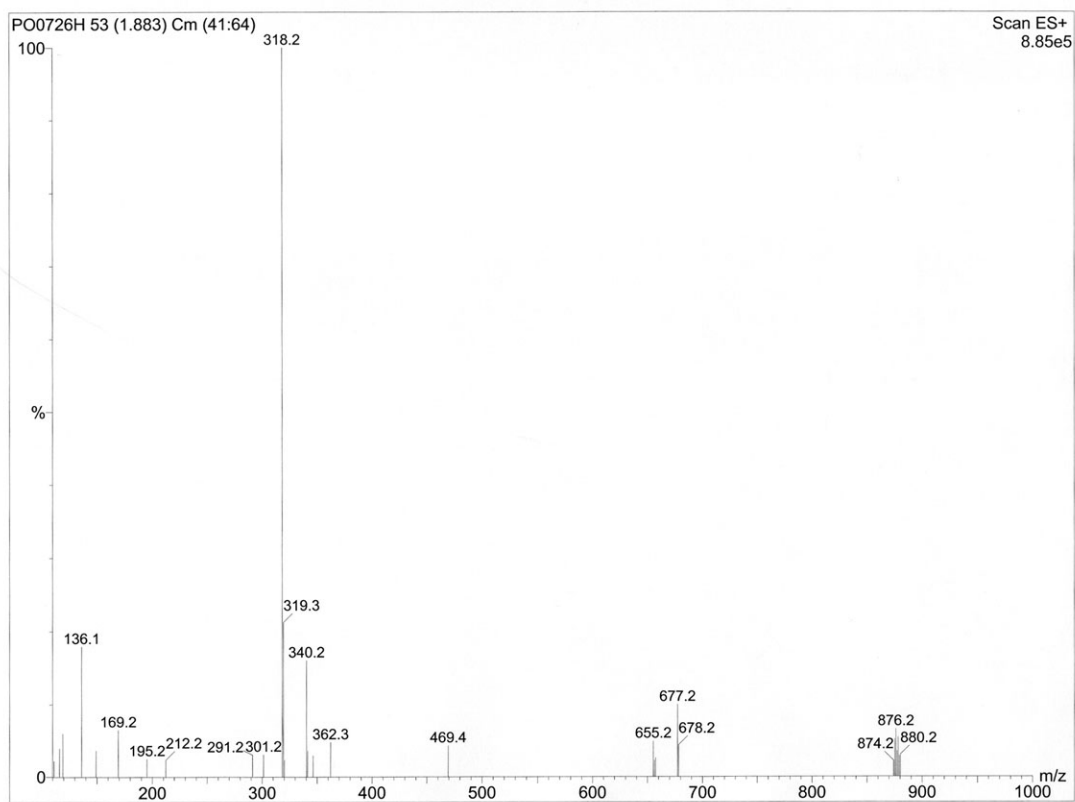


Figure 24. ESI mass spectrum of the benzidine adduct

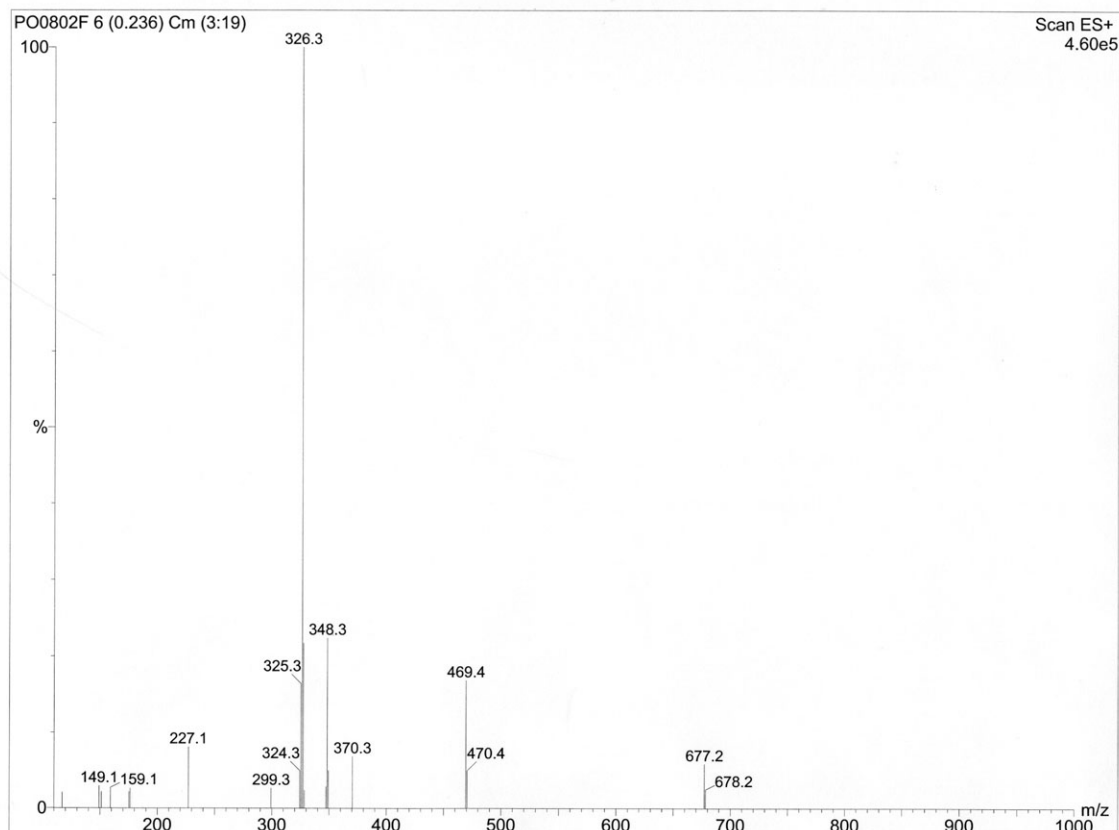


Figure 25. ESI mass spectrum of the benzidine-d₈

The ESI mass spectrum for the 2-aminofluorene adduct, and that of its deuterated form are seen in Figures 26. and 27. respectively. In all cases the $[M+H]^+$ ion of the adduct is clearly the most abundant ion present. In the case of the authentic adducts it is the intensity of this ion that is monitored in order to optimize ionization.

In order to achieve the maximum sensitivity for our method it was necessary to find which MS/MS conditions produce the strongest signal for detection. The mass spectrometer has several parameters that must be tuned to insure that the analyte is completely ionized and fragmented. Most of these parameters, including the skimmer cone voltage, can be optimized by computer ramping programs. Two parameters that must be manually optimized are the capillary ionization voltage and the collision voltage

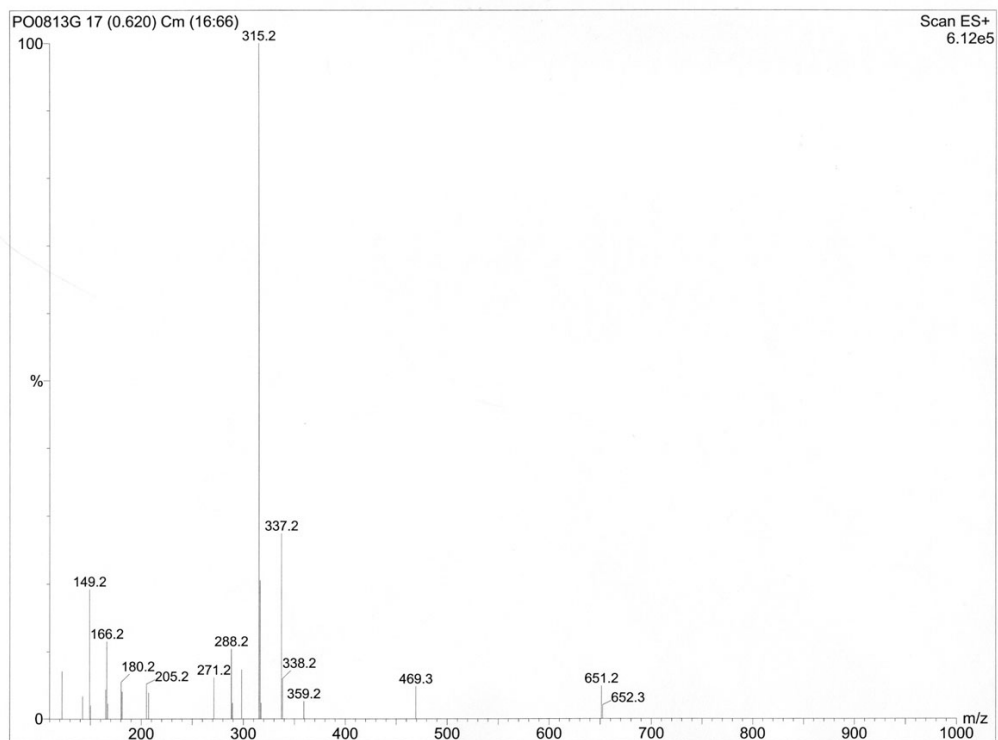


Figure 26. ESI mass spectrum of the 2-aminofluorene adduct

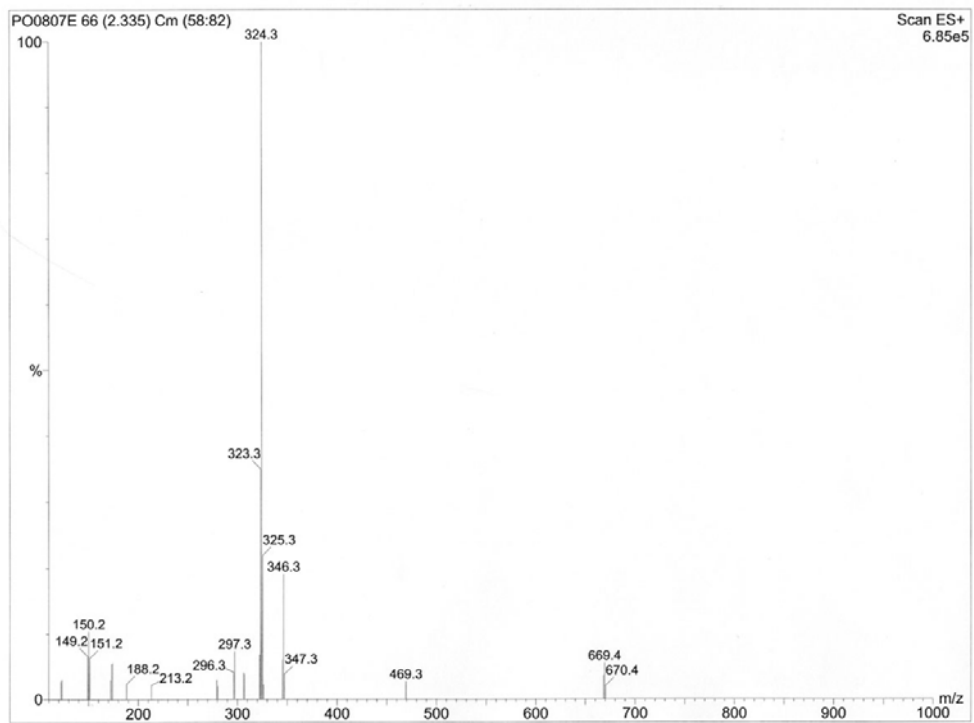


Figure 27. ESI mass spectrum of the 2-aminofluorene-d₉ adduct

used to induce fragmentation. The optimum ionization voltage for the benzidine adduct was found to be 3.2 kV, and that of the 2-aminofluorene adduct was found to be 3.0 kV. The results of the ionization study for the benzidine adduct are shown in Figure 28. The results for 2-aminofluorene are shown in Figure 29. It is possible that at lower voltages

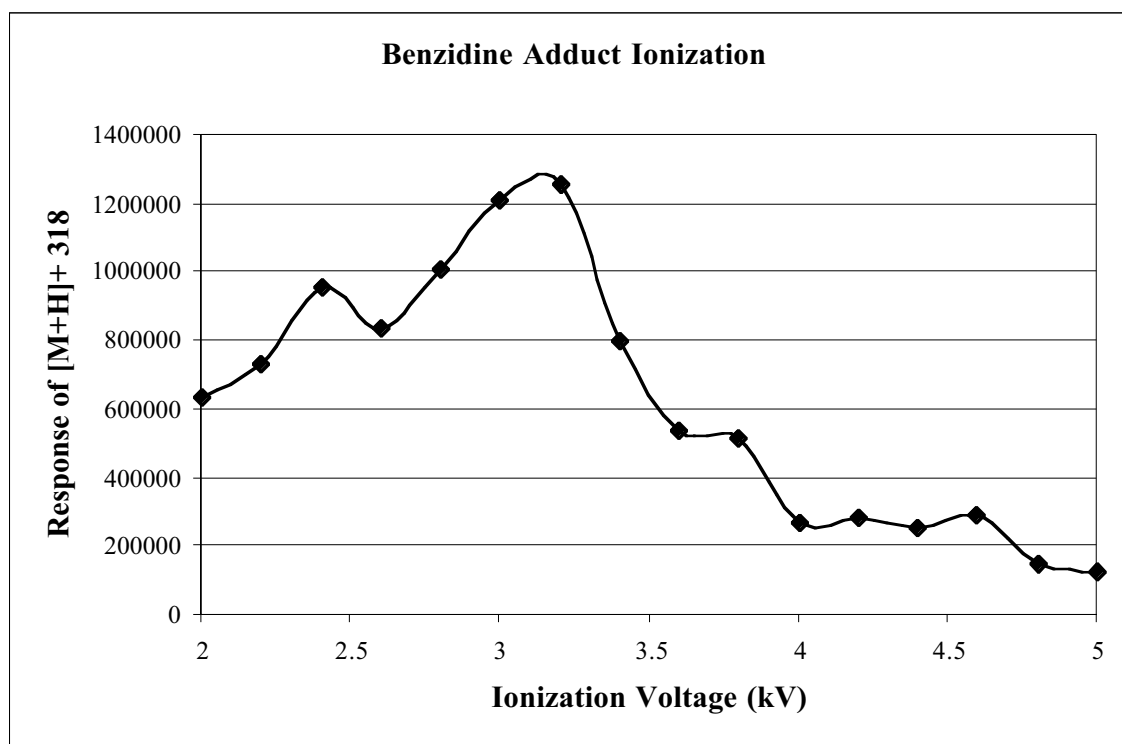


Figure 28. The optimum ionization voltage of the benzidine adduct

reduced sensitivity is due to incomplete sample ionization, while at higher voltages it is due to an arcing effect or sample decomposition.

To insure confirmation of each adduct the transition from its molecular ion to its two most prominent fragments is measured by multiple reaction monitoring. To determine which fragments to monitor and what collision energy is optimal, the first scanning region of the spectrometer was set to pass only the molecular ion and the second region was used for a full scan of fragments. The results of the benzidine adduct collision study are seen in Figure 30.

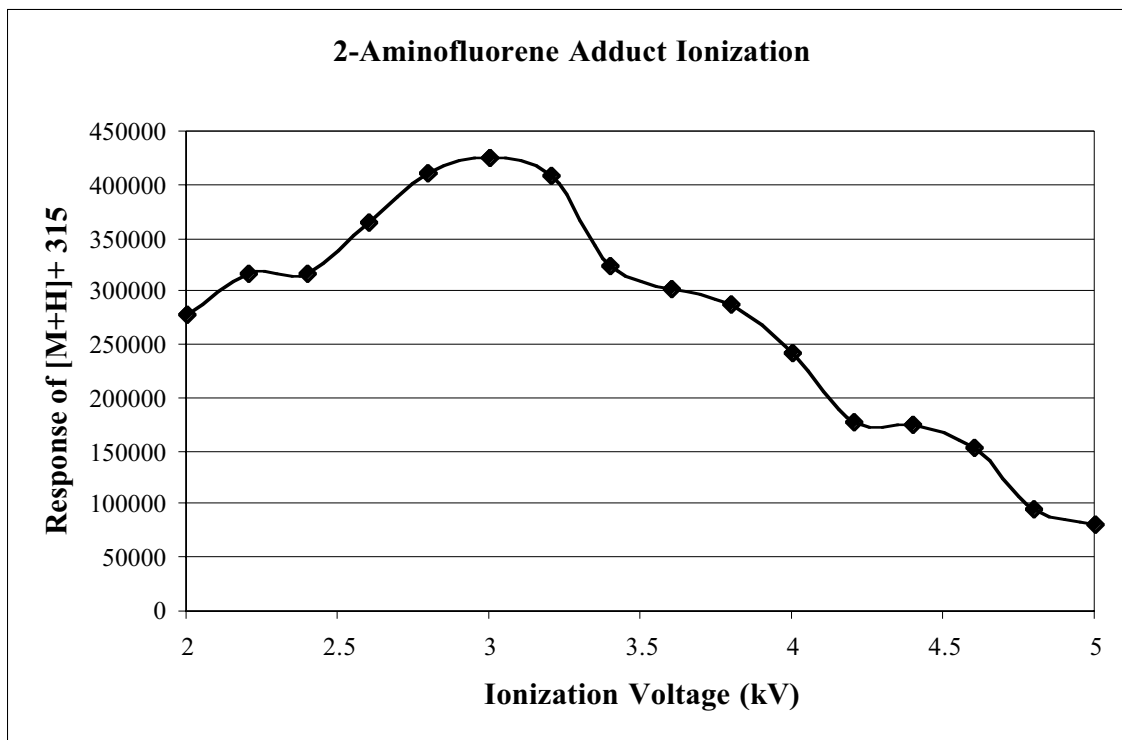


Figure 29. The optimum ionization voltage of the 2-aminofluorene adduct

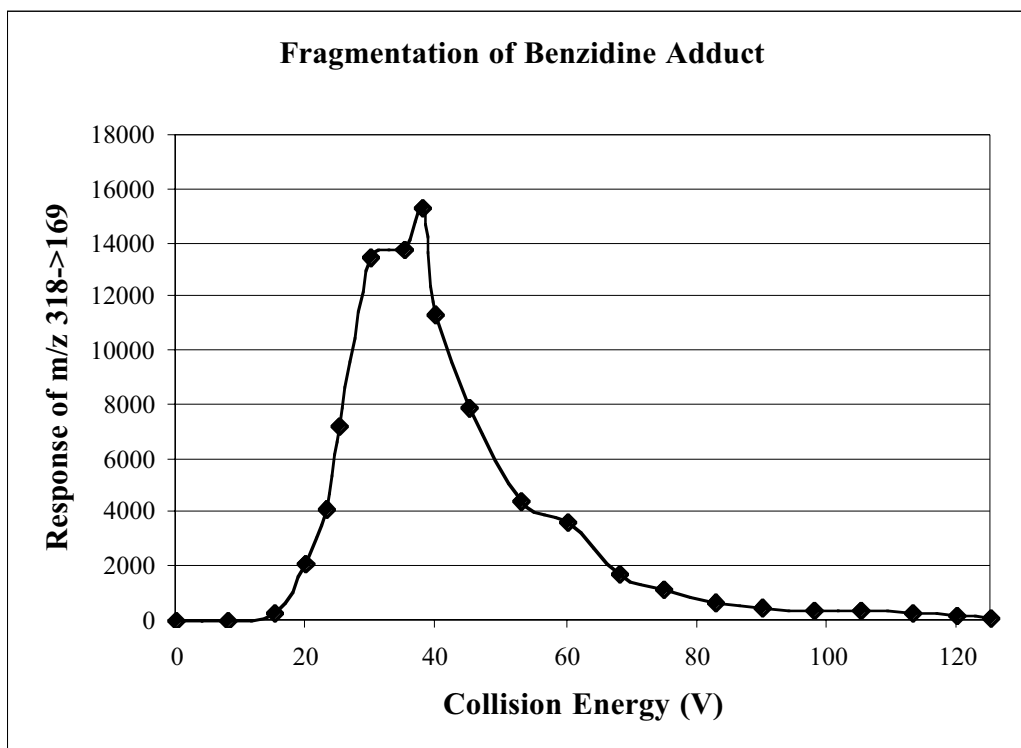


Figure 30. The optimum collision voltage of the benzidine adduct

The optimum collision voltage for the benzidine adduct was found to be 38 V. The ESI MS/MS mass spectrum of the benzidine adduct for these optimized conditions is presented in Figure 31.

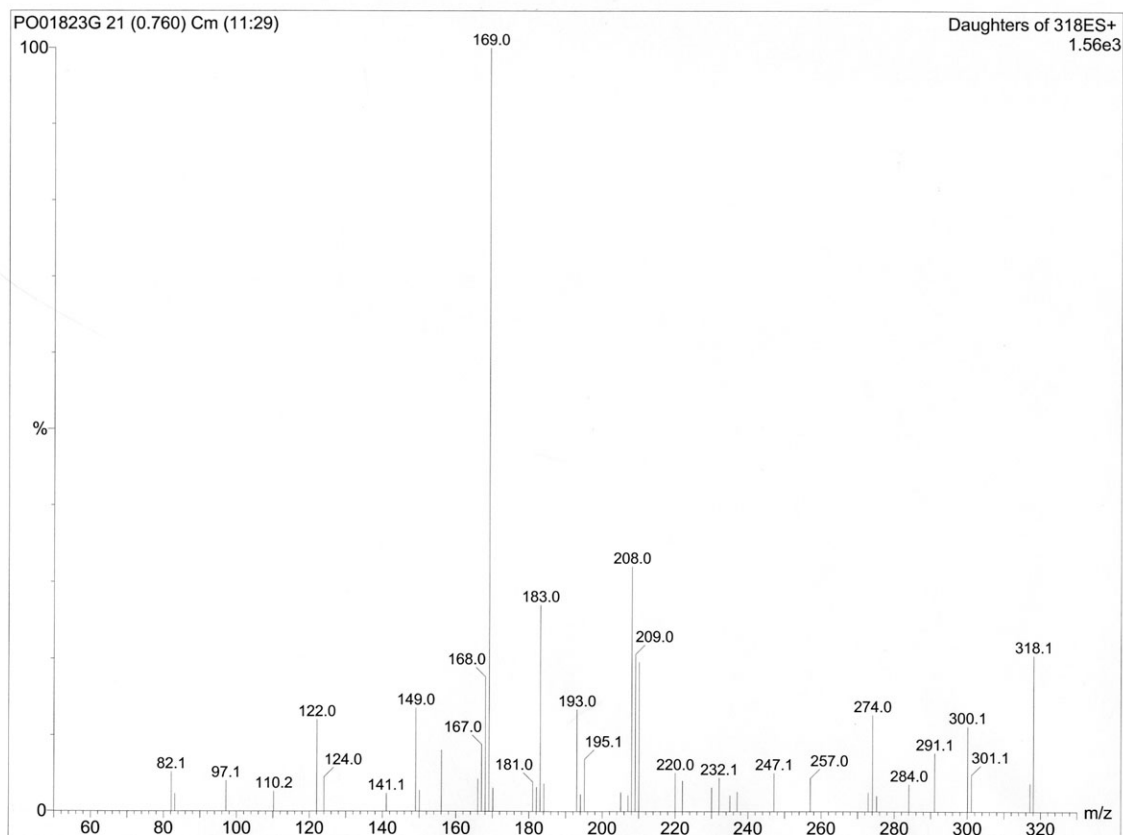


Figure 31. The ESI MS/MS mass spectrum of the benzidine adduct

To determine if these conditions were suitable for fragmentation of the labeled standard, and which of its fragments should be monitored, it was also ran by infusion. The ESI MS/MS mass spectrum of the deuterated benzidine adduct is presented in Figure 32. A logical assignment of these fragments can be seen in Figure 33. Because the fragments of the deuterated adduct differ by a m/z of 7 and not 8 it is possible that during fragmentation one of the deuterium ions is exchanged for a proton.

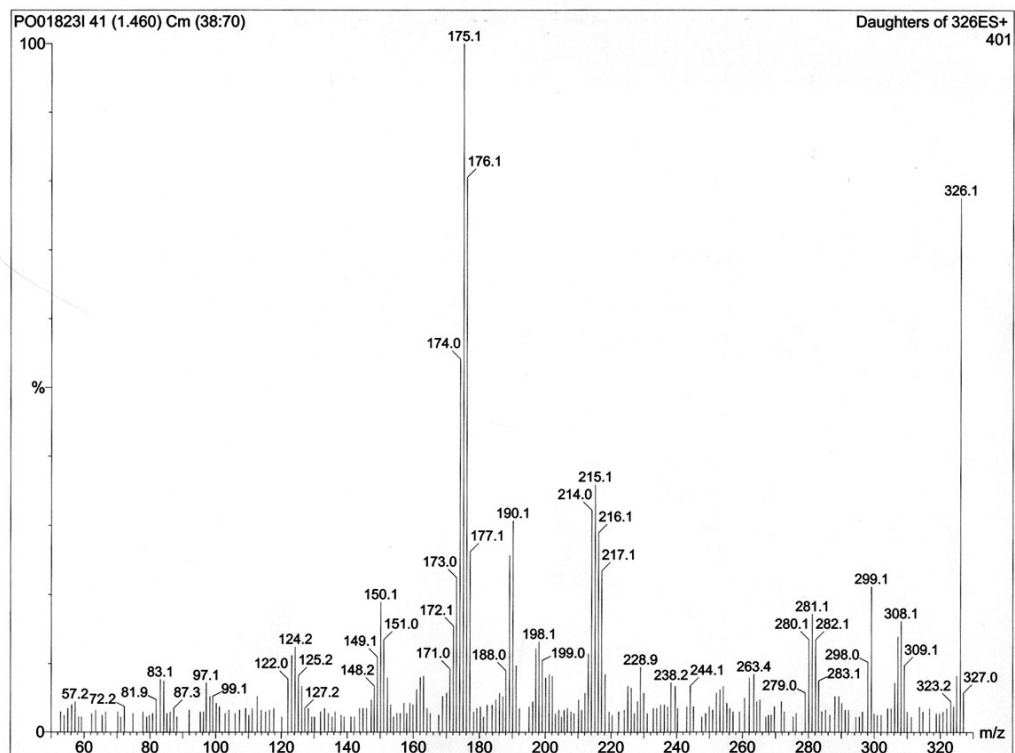


Figure 32. The ESI MS/MS mass spectrum of the benzidine-d₈ adduct

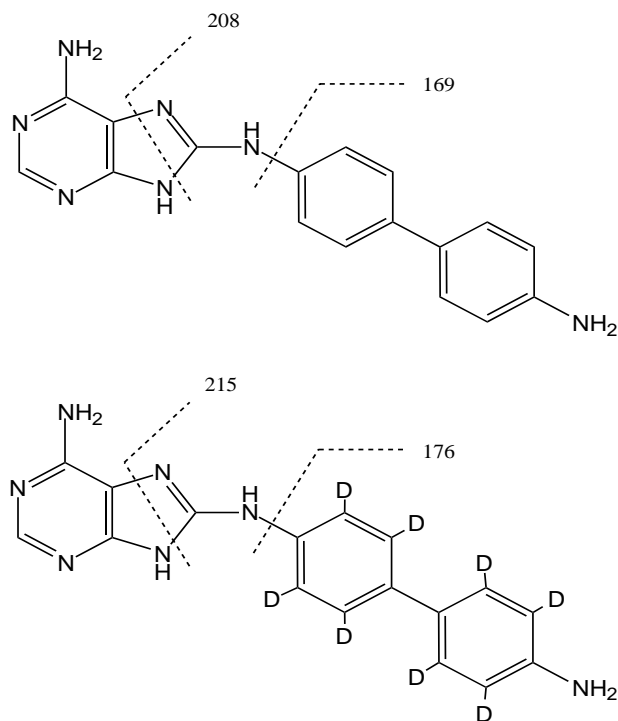


Figure 33. Structural fragments of the benzidine and benzidine-d₈ adducts

The results of the 2-aminofluorene adduct collision study are seen in Figure 34. The optimum collision voltage for the 2-aminofluorene adduct was found to be 70 V. The ESI MS/MS mass spectrum of the 2-aminofluorene adduct under optimized conditions is seen in Figure 35. and that of the deuterated 2-aminofluorene adduct is seen in Figure 36. A logical assignment of these fragments is presented in Figure 37. Again it appears that deuterium/hydrogen exchange occurs during fragmentation as the deuterated fragments differ by a m/z of 8 rather than 9.

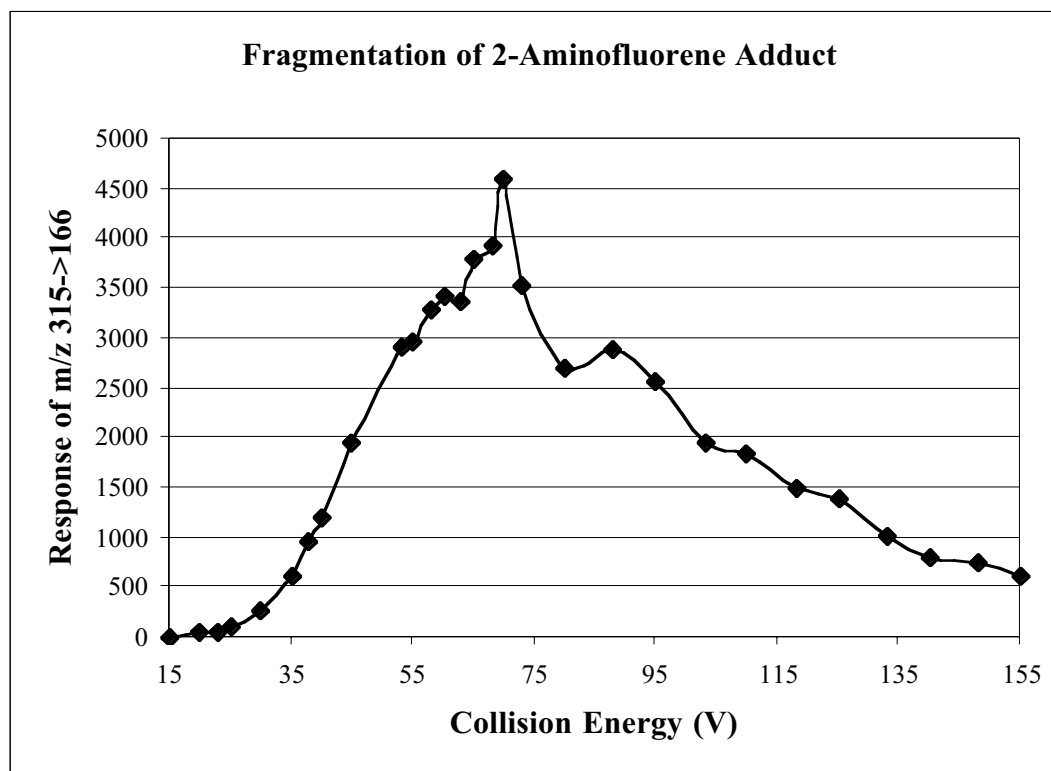


Figure 34. The optimum collision voltage of the 2-aminofluorene adduct

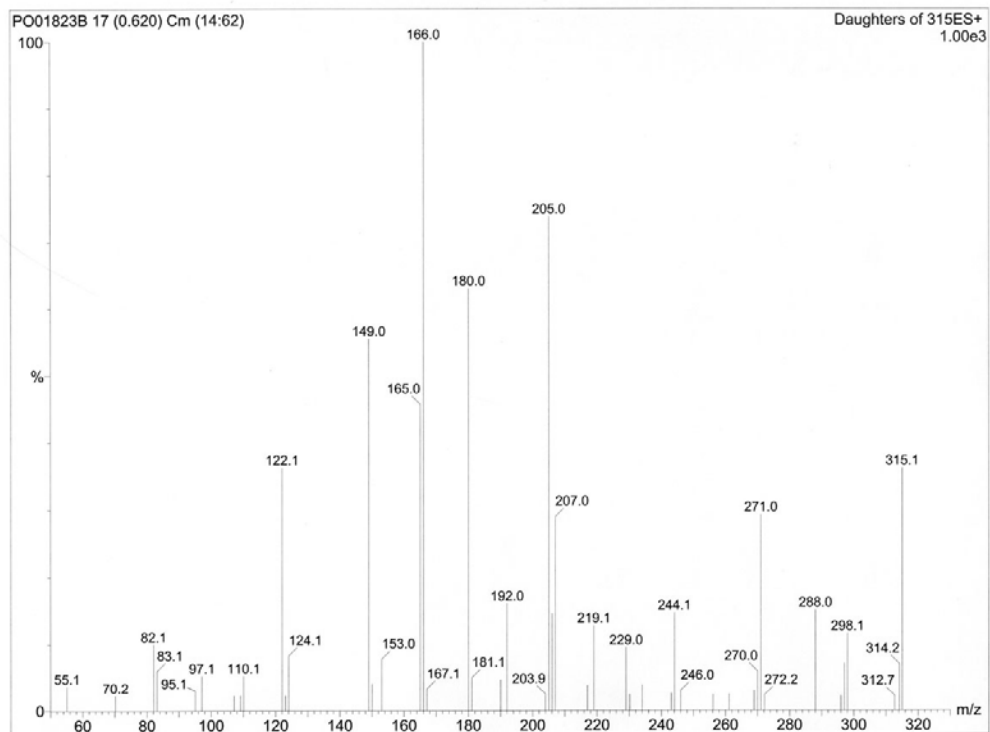


Figure 35. The ESI MS/MS mass spectrum of the 2-aminofluorene adduct

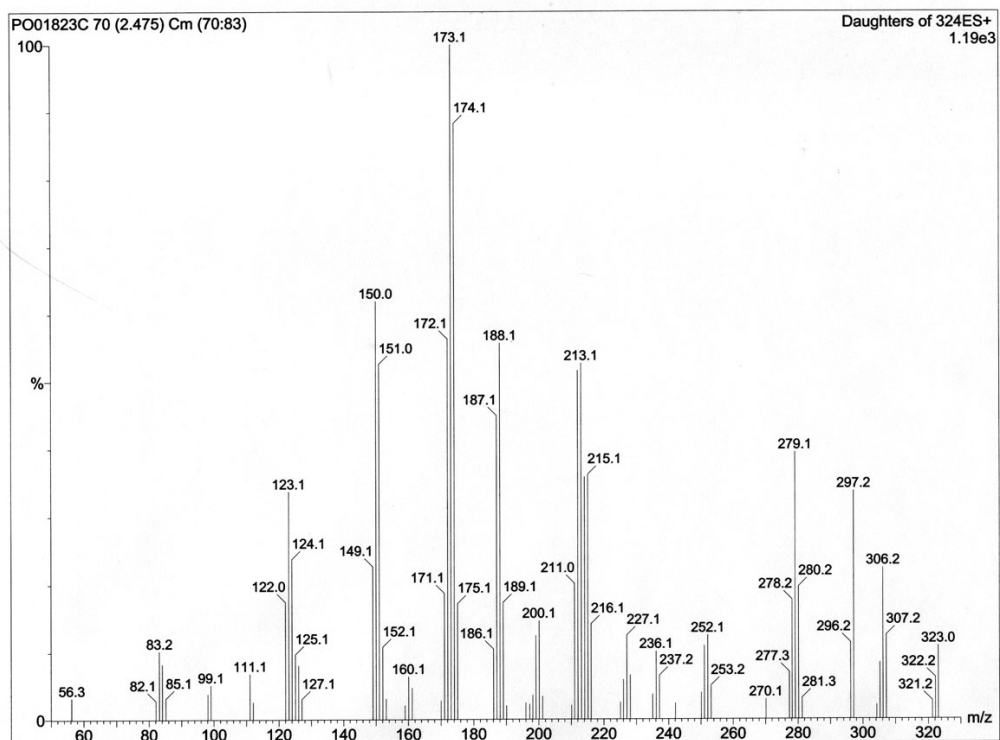


Figure 36. The ESI MS/MS mass spectrum of the 2-aminofluorene-d₉ adduct

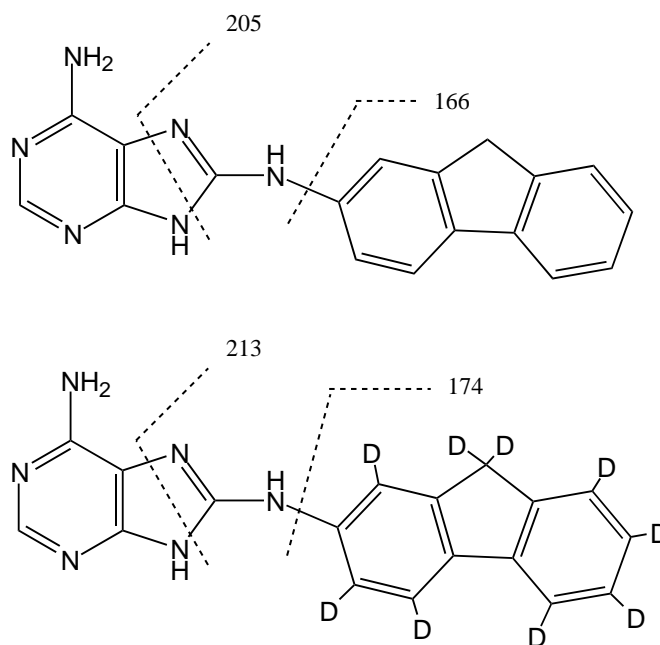


Figure 37. Structural fragments of the 2-aminofluorene and 2-aminofluorene-d₉ adducts

4.3.2 Quantitative LC/MS/MS Detection Method

Before work was begun on the mass spectrometry method, a suitable HPLC separation was found. Electrospray ionization and mass spectrometry place certain requirements on the type of liquid separation technique they can be coupled with. Flow rates must be kept low (<1ml/min.), so that the vacuum of the mass spectrometer can be maintained. Solvents must be low boiling (<120 °C), so that they can be evaporated in the ion source allowing the analyte to enter the mass spectrometer. Nonvolatile buffers that would precipitate and block the source can also not be used. If an acid or base is used to increase the ionization of the analyte it too must be volatile. For our method a flow rate of 1ml/min. was used with a 1:1 split between the column and mass spectrometer. This split reduced the flow rate into the mass spectrometer by half to 0.5ml/min. A linear gradient was developed using water and acetonitrile both containing

0.2% formic acid was developed. With this gradient the benzidine adduct and its deuterated internal standard co-eluted at 12.96 minutes and the 2-aminofluorene adduct and its deuterated internal standard co-eluted at 16.99 minutes.

To improve sensitivity, multiple reaction monitoring was employed for the detection method. This technique improves sensitivity by only detecting specific ions of interest. This is done in primarily two ways. The first is the benefit of removing any interferences that would increase the background noise. The second benefit is that by allowing the scanning regions to spend the entire analysis time on only the analytes of interest, almost none of the signal is lost by spending time scanning other masses. Conformational information is still retained because the ions that are monitored correspond to the specific molecular ions, and their two most abundant fragment ions of the desired analytes. The measurement of these two ions over time produces a chromatogram with peaks corresponding to the concentration of analyte in the sample. Representative chromatograms of the blank and standard solutions are presented in Figure 38. A calibration curve was prepared from triplicate runs of authentic standard solutions over a range of 1 to 32 ng (1, 2, 4, 8, 16, and 32 ng) of adduct on column. Each standard injection also contained 2 ng of deuterated internal standard. Peak height was used rather than area as it gave a more accurate result.

The processed data used to construct the standard curve for the benzidine adduct is presented in Table 5. A linear response versus concentration was determined ($y = 0.8324x - 0.1728$) using the method of least squares with a resulting R^2 of 0.9981 (Figure 39). Most of the standards were accurate. The overall percent accuracy of all standards ranged from 92 to 112 % with the highest percent relative standard deviation of 6.33%.

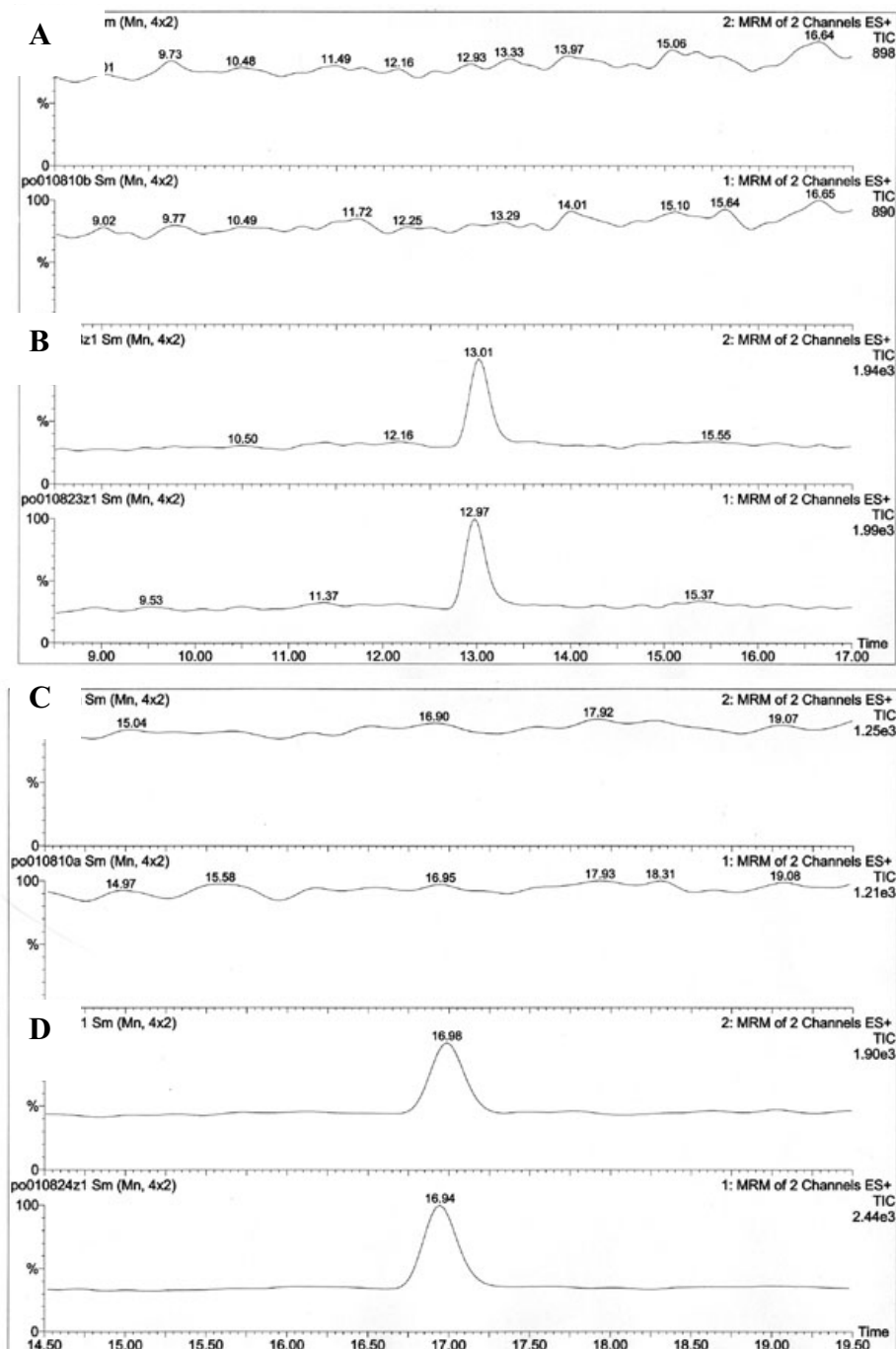


Figure 38. Representative chromatograms of blank and standard solutions of the benzidine and 2-aminofluorene adducts. For each pair the top chromatogram is the authentic standard response and the bottom chromatogram is the internal standard response. A. Benzidine adduct blank B. 1 ng Benzidine standard C. 2-aminofluorene blank D. 1 ng 2-aminofluorene standard

Table 5. Benzidine Adduct Standard Data

Ng Adduct On Column	Ng Adduct Calculated	% Accuracy	Average % Accuracy \pm Standard Deviation	% RSD
1.00	1.11	111	110 \pm 1.2	1.10
1.00	1.10	110		
1.00	1.08	108		
2.00	2.01	100	105 \pm 6.6	6.33
2.00	2.03	102		
2.00	2.24	112		
4.00	3.83	96	102 \pm 5.8	5.71
4.00	4.29	107		
4.00	4.10	102		
8.00	8.01	100	101 \pm 1.0	1.03
8.00	8.15	102		
8.00	8.16	102		
16.00	14.92	93	93 \pm 1.7	1.85
16.00	14.67	92		
16.00	15.22	95		
32.00	32.04	100	101 \pm 1.3	1.24
32.00	32.62	102		
32.00	32.82	103		

The limit of detection was pg (69 fmole) (S/N = 3) and the limit of quantitation was 73 pg (230 fmole) (S/N = 10).

The processed data used to construct the standard curve for the 2-aminofluorene adduct is presented in Table 6. A very linear response versus concentration was determined ($y = 0.5449x + 0.1454$) using the method of least squares with a resulting R^2 of 0.9996 (Figure 40). The overall percent accuracy of all standards ranged from 89 to 106 % with the highest percent relative standard deviation being 6.06 %. The limit of detection was 51 pg (162 fmole) (S/N = 3) and the limit of quantitation was 171 pg (543 fmole) (S/N = 10).

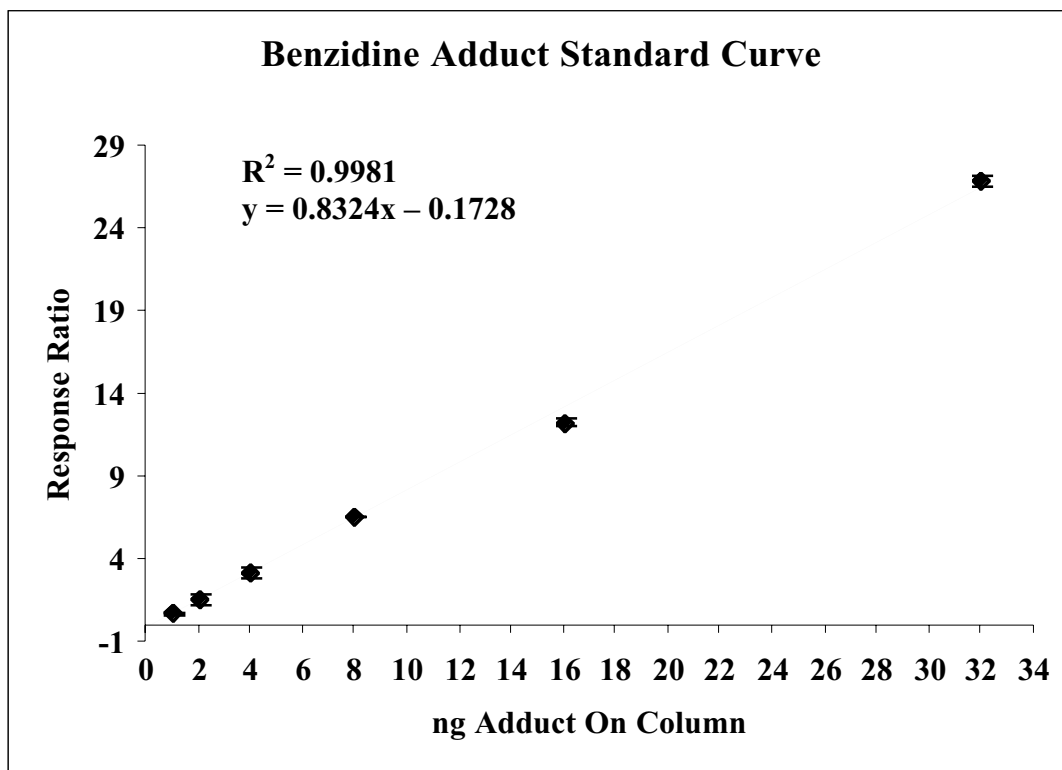


Figure 39. The standard curve for the benzidine adduct

Table 6. 2-Aminofluorene Adduct Standard Data

Ng Adduct On Column	Ng Adduct Calculated	% Accuracy	Average % Accuracy \pm Standard Deviation	% RSD
1.00	0.93	93	93 \pm 4.2	4.49
1.00	0.89	89		
1.00	0.97	97		
2.00	2.13	106	102 \pm 6.2	6.06
2.00	1.89	95		
2.00	2.08	104		
4.00	4.18	105	101 \pm 3.5	3.46
4.00	3.91	98		
4.00	4.01	100		
8.00	8.52	106	106 \pm 1.1	1.04
8.00	8.35	104		
8.00	8.45	106		
16.00	16.42	103	100 \pm 3.5	3.45
16.00	16.35	102		
16.00	15.48	96		
32.00	31.36	98	100 \pm 1.5	1.49
32.00	31.89	100		
32.00	32.31	101		

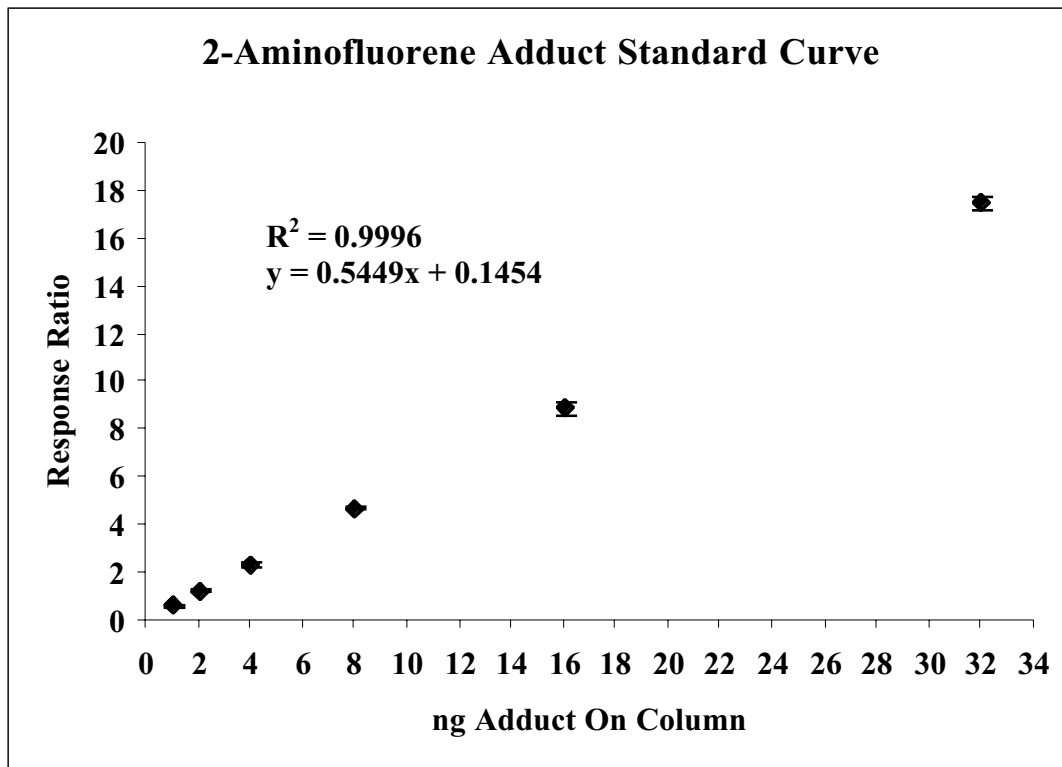


Figure 40. The standard curve for the 2-aminofluorene adduct

4.3.3 Spike and Recovery Studies

In order to demonstrate the method validity, adduct stability, and procedure efficiency two separate spike and recovery studies were performed. The first determined the adduct stability to acid hydrolysis and the efficiency of Sep-Pak C18 cartridge recovery. The second spike and recovery study demonstrated the effect of a biological matrix, in this case DNA, on adduct recovery, and determined if any natural interferences were present.

For the stability and recovery study the internal standard was added to the evaporated residue after the Sep-Pak filtration in order to insure a constant value. The

recovery of the benzidine adduct spike varied greatly ranging from 39-95 % recovery, with an average of 68% (Table 7).

Table 7. Stability Study of the Benzidine Adduct

Ng Benzidine Adduct Spike	Ng Benzidine Adduct Calculated	Recovery (%)	Average Recovery
1.00	0.90	90	91
1.00	0.91	91	
5.00	4.69	94	93
5.00	4.58	92	
10.00	9.21	92	91
10.00	8.98	90	
20.00	18.21	91	92
20.00	18.44	92	

The recovery of the 2-aminofluorene adduct spike also varied greatly ranging from 40-119 % recovery, with an average of 78% (Table 8).

Table 8. Stability Study of the 2-Aminofluorene Adduct

Ng 2-Aminofluorene Adduct Spike	Ng 2-Aminofluorene Adduct Calculated	Recovery (%)	Average Recovery
1.00	0.96	96	97
1.00	0.97	97	
5.00	4.75	95	96
5.00	4.81	96	
10.00	9.69	97	96
10.00	9.48	95	
20.00	19.24	96	96
20.00	19.07	95	

The fact that all samples were hydrolyzed for the same amount of time and eluted with the same amount of methanol make it unlikely that this variance is due to standard

decomposition or Sep-Pak retention. It is more likely that the 150 μ L of DMSO used to redissolve the sample was inefficient at taking up the entire sample. The 3 mL eluents were collected in 4 mL glass vial and evaporated under ultra pure nitrogen. Although after the DMSO was added, all the vials were vigorously vortexed for 30 seconds, it is possible that random amount of the spike had absorbed near the rim of each vial preventing it from reincorporating into the sample. In order to overcome this the second spike and recovery study concentrated each sample in a 1.5 mL vial in 1 mL batches including a 1 mL rinse of fresh methanol. Representative chromatograms of blank and spiked samples are presented in Figure 41.

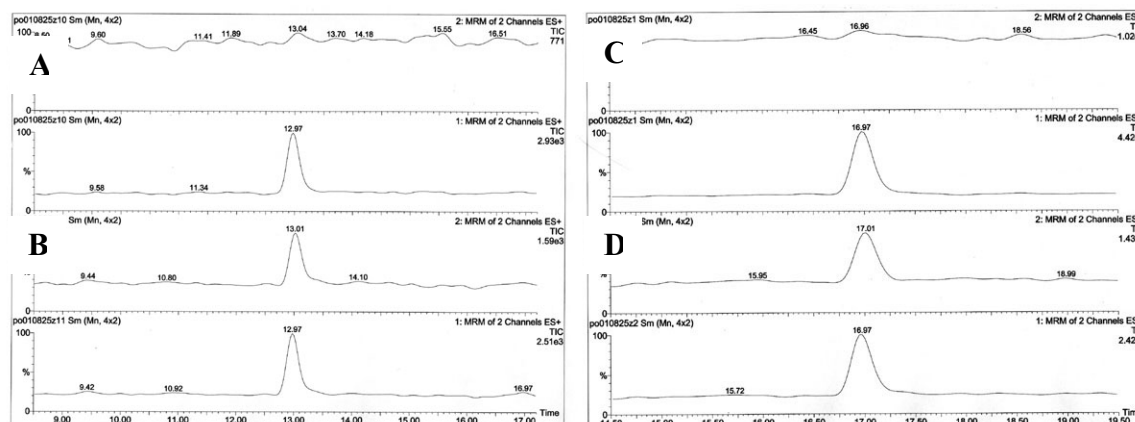


Figure 41. Representative chromatograms of blank and recovery/stability samples. For each pair the top chromatogram is the authentic spike response and the bottom chromatogram is the internal standard response. A. Benzidine adduct blank B. 1 ng Benzidine spike C. 2-aminofluorene blank D. 1 ng 2-aminofluorene spike

The results for the DNA spike and recover experiment samples are much improved over the stability/efficiency study. Average recoveries were much higher and contained much less variance. The recovery of the benzidine adduct in the DNA samples ranged from 96-114 % recovery, with an average of 105% (Table 9).

Table 9. DNA Recovery Study of the Benzidine Adduct

Ng Benzidine Adduct Spike	Ng Benzidine Adduct Calculated	Recovery (%)	Average Recovery
1.00	0.96	96	98
1.00	0.99	99	
5.00	5.31	106	108
5.00	5.49	110	
10.00	10.18	102	108
10.00	11.43	114	
20.00	21.36	107	106
20.00	20.95	105	

The recovery of the 2-aminofluorene adduct in the DNA samples ranged from 95-117 % recovery with an average recovery of 105 % (Table 10).

Table 10. DNA Recovery Study of the 2-Aminofluorene Adduct

Ng 2-Aminofluorene Adduct Spike	Ng 2-Aminofluorene Adduct Calculated	Recovery (%)	Average Recovery
1.00	0.95	95	97
1.00	0.99	99	
5.00	5.71	114	108
5.00	5.09	102	
10.00	11.65	117	110
10.00	10.31	103	
20.00	20.06	100	105
20.00	21.86	109	

This improved recovery is due to two primary factors. The first is that the internal standard underwent the entire sample procedure along with the non-labeled authentic adduct spike. Thus if any material was lost a proportional amount of the internal standard was also lost to make up for it. The second factor, which may be even more significant is

that the evaporation and re-concentration step was performed in smaller vials allowing for complete re-incorporation of the sample residue.

Representative chromatograms of blank and 1 ng adduct spikes in DNA samples are presented in Figure 42.

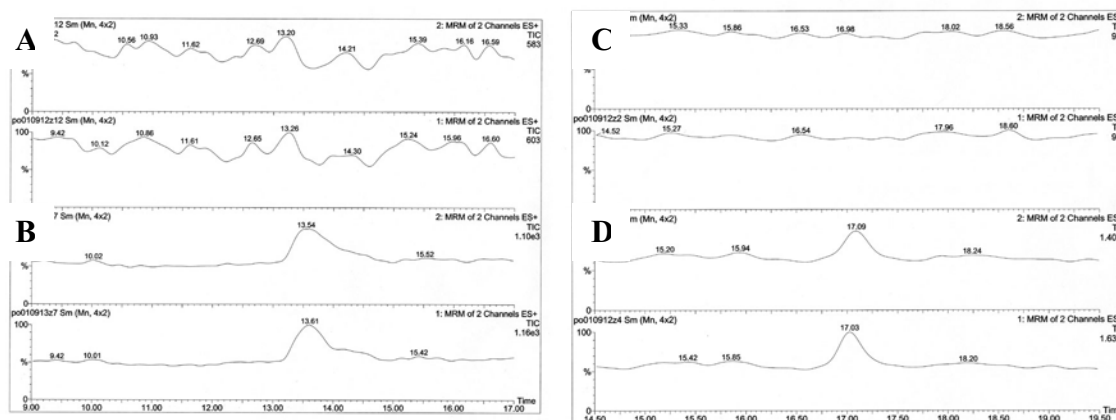


Figure 42. Representative chromatograms of blank DNA and DNA spike samples. For each pair the top chromatogram is the authentic spike response and the bottom chromatogram is the internal standard response. A. Benzidine adduct blank B. 1 ng Benzidine spike C. 2-aminofluorene blank D. 1 ng 2-aminofluorene spike

From Figure 42. B. it can be seen that the retention time of the benzidine adduct has been shifted from 12.99 minutes to 13.58 minutes. This is due to the vast abundance of non-adducted nucleotides from the DNA sample that coelute in this region. The presence of this interference has changed the partitioning of the analyte between the mobile and stationary phases. This interference can be seen by the online UV detector set at 300nm between the HPLC column and the mass spectrometer in Figure 43. The large interference peak shifts the retention time of the benzidine adduct by altering its partition coefficient between the stationary and mobile phase. Although the retention time has changed due to the interference, the sensitivity of the MS detector is not affected. This can be seen in the low background of Figure 43. B. Altering the HPLC gradient so that

the benzidine adduct would not coelute with the interference would produce a consistent retention time. It may also be possible to remove the interference during the C₁₈ concentration step by performing additional washes before eluting.

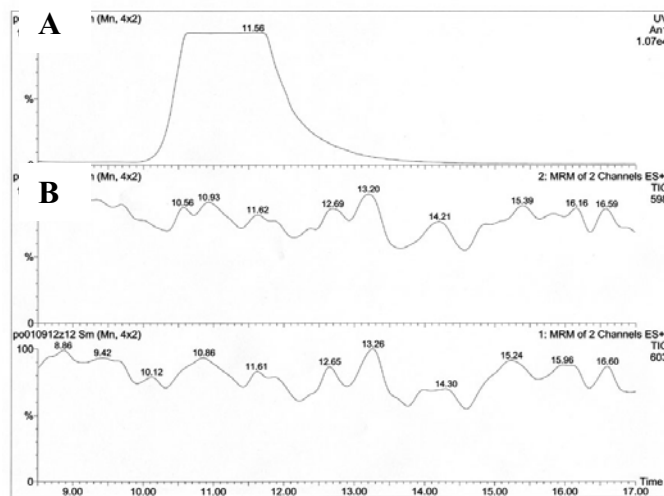


Figure 43. UV and benzidine adduct MRM response of a DNA blank. A. The UV response at 300 nm. B. The MRM response of the benzidine adduct. The top chromatogram corresponds to the response of the authentic adduct and the bottom corresponds to that of the internal standard.

In conclusion, we have developed a sensitive method for the detection of benzidine and 2-aminofluorene C8 adenine adducts in DNA with random efficiency. The average limit of detection was ~ 100 fmole which would correspond to one adduct in $\sim 6 \times 10^7$ bases for a 4 mg DNA sample, well within physiologically relevant levels. Our sample preparation procedure has an average recovery of 105 %.

CHAPTER 5 CONCLUSION

In summary we have developed a new synthetic method involving a palladium catalyst for the synthesis of C8-adenine arylamine adducts. This method was used to prepare adduct of several arylamine in yields of 25-84 %. Some of the adducts were then used as standards in LC/MS/MS detection method. A standard curve was constructed for the benzidine C8-adenine adduct having an $R^2 = 0.9981$ with a limit of detection of 22 pg (69 fmole) (S/N = 3), and a limit of quantitation of 73 pg (230 fmole) (S/N = 10). A standard curve was constructed for the 2-aminofluorene C8-adenine adduct having an $R^2 = 0.9996$ with a limit of detection of 51 pg (162 fmole) (S/N = 3), and a limit of quantitation of 171 pg (543 fmole) (S/N = 10). This method was then validated by two spike and recovery studies that demonstrated adduct stability, detection efficiency, and the lack of interferences.

Our synthetic method is a great improvement over existing techniques to prepare adducts. Other methods typically result in yields of less than 5 % and require preparative HPLC. Our method allows for the preparation of adducts in a few easy steps on a scale readily sufficient for characterization by NMR. Although our method was unsuccessful for producing C8-guanine adducts it is likely that it could be, with the incorporation of extra protective and deprotective steps. As it is, the significance of minor C8-adenine adducts compared to more common C8-guanine adducts, is much less studied, in part due to the difficulties of preparing standards. Our method overcomes this obstacle. Our method is also likely to be useful in preparing adducts of substituted methylene dianiline compounds that are known to produce C8-adenine adducts predominantly.

Our LC/MS/MS method has shown to be very sensitive, capable of detecting one adduct per $\sim 6 \times 10^7$ bases of DNA. This sensitivity is sufficient for the detection of adducts at physiological levels. This method is applicable for quantifying C8-adenine adducts of benzidine and 2-aminofluorene from *in vitro* and *in vivo* studies. It could be used to correlate arylamine exposure to adduct formation frequency, or the kinetics of adduct formation and repair. It could also be used as a diagnostic method to screen for DNA damage in environmental and industrial settings. By slightly changing the multiple reaction monitoring of the mass spectrometer this method could be modified to detect other arylamine adduct isomers, though quantitation would not be as precise. Current work is being conducted to improve recovery reproducibility.

Overall this work has contributed to the study of arylamine carcinogenicity. Both our synthetic and analytical methods have numerous applications for future studies in this field. The possibilities of heteroatom organometallic catalysis, and LC/MS/MS are virtually limitless and research in both areas should be continued in order to better understand the world around us, and our interaction in it.

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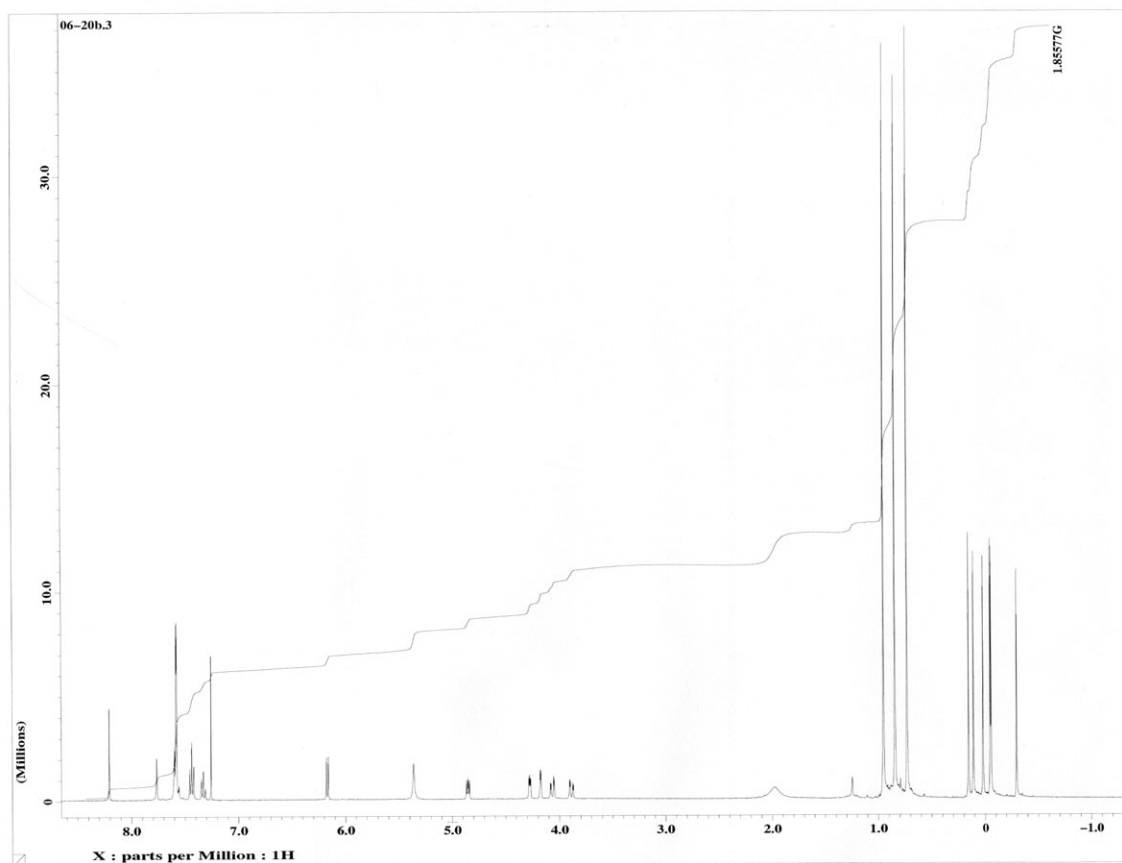
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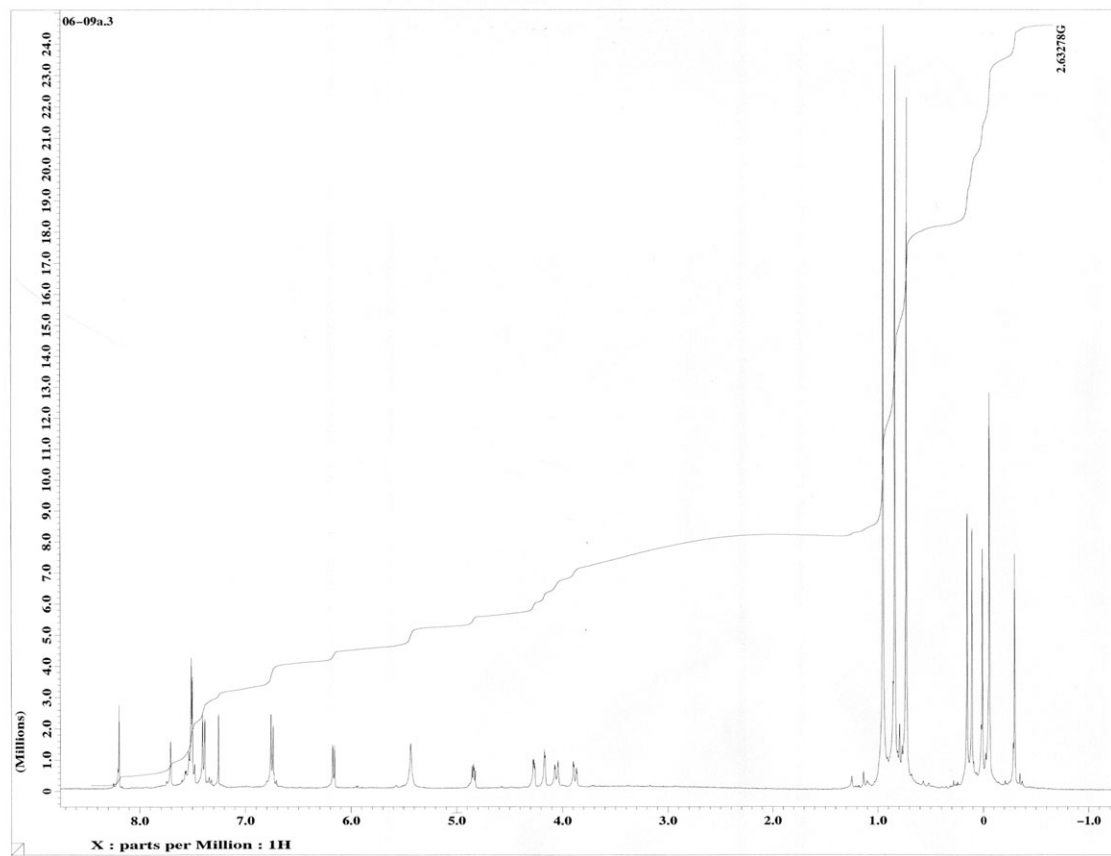
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APPENDIX

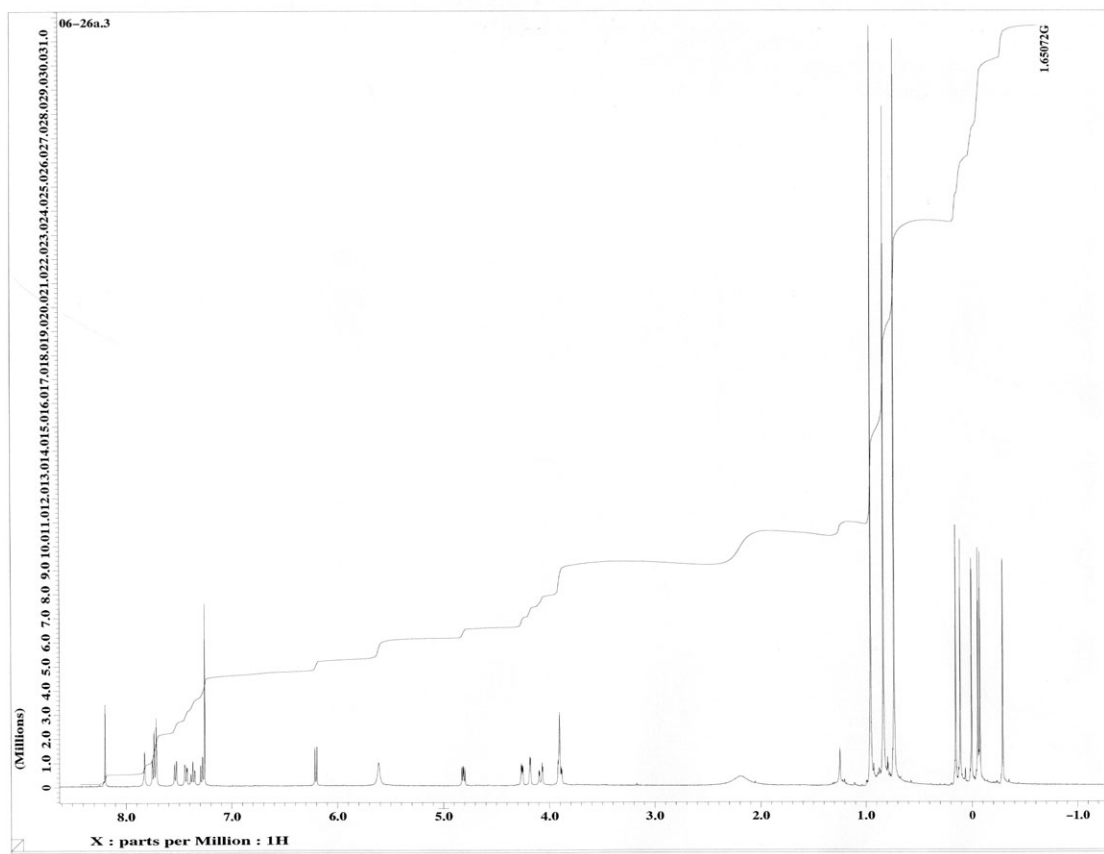
H¹ NMR Spectra



N-(2',3',5'-tris-O-*tert*-butyldimethylsilyladenosin-8-yl)-4-aminobiphenyl



N-(2',3',5'-tris-O-*tert*-butyldimethylsilyladenosin-8-yl)-benzidine



N-(2',3',5'-tris-O-*tert*-butyldimethylsilyladenoin-8-yl)-2-aminofluorene

VITA

Philip Dmitri Olsen was born March 23, 1973 in Fontana, California, near Riverside. At the age of eleven he moved with his family to the city of Los Angeles. After graduating from Palisades High School in 1991, he went on to the University of California at Santa Cruz. He received his bachelors of science degree in chemistry in 1995. He then went to Louisiana State University to study environmental analytical chemistry with an emphasis on toxicology. He is currently a candidate for a Doctor of Philosophy degree in Chemistry.